Molecular analyses of bacterial communities in drinking water and biofilms – seasonal dynamics, viability and community composition of cold and hot water

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Publikationen

1. Henne K., L. Kahlisch, J. Draheim, I. Brettar, und M. G. Höfle. 2008. Polyvalent fingerprint based molecular surveillance methods for drinking water supply systems. Water Sci. Technol: Water Supply 8:527.

2. Henne K., L. Kahlisch, I. Brettar, und M. G. Höfle. 2012. Comparison of Structure and Composition of Bacterial Core Communities in Mature Drinking Water Biofilms and Bulk Water of a Local Network. Appl. Environ. Microbiol. AEM.06373-11; published ahead of print 2 March 2012

3. Kahlisch L., K. Henne, L. Groebe, J. Draheim, M. G. Höfle, und I. Brettar. 2010. Molecular analysis of the bacterial drinking water community with respect to live/dead status. Water Sci. Technol 61:9–14.

4. Kahlisch L., K. Henne, J. Draheim, I. Brettar, und M. G. Hofle. 2010. High-resolution in situ genotyping of Legionella pneumophila populations in drinking water by Multiple-Locus Variable-Number of Tandem Repeat Analysis (MLVA) using environmental DNA. Appl. Environ. Microbiol. AEM.00416–10.

5. Kahlisch L., K. Henne, L. Gröbe, I. Brettar, und M. Höfle. 2012. Assessing the Viability of Bacterial Species in Drinking Water by Combined Cellular and Molecular Analyses. Microb Ecol. 63:383–397.

Tagungsbeiträge

1. Henne K, L. Kahlisch, J. Draheim, I. Brettar, M. G. Höfle. Seasonal cycle and source water dependence of the bacterial community of drinking water as revealed by polyvalent fingerprint based molecular analysis. (Vortrag) Jahrestagung der Deutschen Gesellschaft für Limnologie (DGL). Konstanz, Deutschland, 2008.

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"Science (...) is like sex: sure, it may give some practical results, but that's not why we do it."

nach Richard P. Feynman

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Summary

Up to now, drinking water maintained its role as the most important consumer good worldwide. Therefore the drinking water quality has to be of pronounced importance public health. Although water treatment and disinfection measures aim at eliminating all harmful and pathogenic mircroorganisms, the water quality at the customer tap does not necessarily provide the same quality as the drinking water leaving the waterworks facility and still provides an environment for a regrowing diverse microflora. The objective of the present thesis was to get insights into the bacterial drinking water communities and their associated habitats such as drinking water biofilm and hot drinking water. Therefore, it is of relevance, which environmental factors are influencing the presence and activity of the members of these communities and what interactions exist between these communities.

In the first two parts of the thesis we investigated the bacterial dynamics in the drinking water of Braunschweig, Germany, over the timeframe of 18 month. Using single stranded conformation polymorphism (SSCP) fingerprints and sequencing major bands we analysed the seasonal dynamics of the community composition and its activity of cold and hot drinking water sampled at the campus of the Helmholtz Centre for Infection Research (HZI) in relation to meteorological data of the catchment area. For cold drinking water it was demonstrated that the precipitation was the major impact influencing the amount and activity of bacteria. The hot drinking was mainly composed of thermophilic and thermotolerant bacteria. In the third part a detailed analysis of the bacterial community in biofilms and corresponding bulk water was done concentrating on the over 20 year old drinking water supply system (DWSS) of the HZI. The overall community structure of the bacteria in the bulk water was the same across the city of Braunschweig whereas the bacteria in all biofilm samples showed a highly different structure with no overlap to the phylotypes observed in bulk water. Biofilm communities sampled on nearby sampling points showed similar communities in spite of different support materials. In the fourth part we investigated the question which bacterial species are present in drinking water. Therefore a combination of Live/Dead-staining, Fluorescence Activated Cell Sorting (FACS) and community fingerprinting was used for the analysis of a set of drinking water samples. The developed approach enabled monitoring of the bacterial drinking water community and assessment of the physiological state of taxonomic groups of interest.

Zusammenfassung

Trinkwasser hat sich bis heute seine Rolle als weltweit wichtigstes Lebensmittel bewahrt, daher ist die Trinkwasserqualität von herrausragender Wichtigkeit für das Gesundheitswesen. Obwohl die üblichen Wasseraufbereitungsmaßnahmen darauf abzielen, alle schädlichen und pathogenen Mikroorganismen zu eliminieren, erreicht die Wasserqualität beim Endverbraucher nicht zwingendermaßen dieselbe Qualität wie das Wasser, das gerade die Wasseraufbereitung verlassen hat. Das Trinkwasser bietet nämlich immer noch einen Lebensraum für eine nachwachsende, vielfältige Mikroflora. Die Zielsetzung der vorliegenden Dissertation war, Einblicke in die bakteriellen Gemeinschaften von Trinkwasser und seinen assoziierten Habitaten wie Trinkwasserbiofilme und Heißwasser zu bekommen. Dazu war die Fragestellung, welche Umweltfaktoren die Präsenz und die Aktivität der Mitglieder dieser bakteriellen Gemeinschaften beeinflussen und welche Interaktionen zwischen diesen Gemeinschaften existieren.

In den ersten beiden Teilen der Dissertation untersuchten wir die bakterielle Dynamik im Braunschweiger Trinkwasser über einen Zeitraum von 18 Monaten. Mithilfe von Einzelstrang-Konformationspolymorphismus-Fingerprints (SSCP) und dem Sequenzieren der Hauptbanden wurde die saisonale Dynamik der Zusammensetzung der bakteriellen Gemeinschaft und ihrer Aktivität im kalten und heißem Trinkwasser untersucht, das auf dem Campus des Helmholtzzentrums für Infektionsforschung (HZI) entnommen wurde. Die gewonnen Daten wurden in Relation zu den meteorologischen Daten des Einzugsgebiets der Trinkwassertalsperren gesetzt. Es wurde für das kalte Trinkwasser gezeigt, dass die Anzahl der Bakterien und Ihre Aktivität hauptsächlich durch den Niederschlag beeinflußt wurde. Das heiße Trinkwasser bestand hauptsächlich aus thermophilen und thermotoleranten Bakterien. Im dritten Teil wurde eine detaillierte Analyse der bakteriellen Gemeinschaft in Trinkwasser-Biofilmen und dem dazugehörigen Wasser untersucht. Hierbei wurde sich auf das über 20 Jahre alte Trinkwasserversorgungssystem des HZI konzentriert. Die allgemeine Struktur der bakteriellen Gemeinschaft war überall im Stadtgebiet die gleiche, während alle Biofilme eine eigene, einzigartige Struktur aufwiesen, ohne gemeinsame Phylotypen zwischen dem Wasser und den Biofilmen. Trotz unterschiedlichen Oberflächenmaterials wiesen benachbarte Biofilme ähnliche bakterielle Gemeinschaften auf. Im vierten Teil wurde untersucht, welche Bakterien lebendig im Trinkwasser zu finden sind. Um diese Frage zu beantworten, wurde ein kombinierter Ansatz aus Lebend/Tot-Färbung und Durchflußzytometrie verwendet. Dieser Ansatz ermöglichte das Monitoring der bakteriellen Gemeinschaft im Trinkwasser und eine Einschätzung des physiologischen Zustandes der bedeutenden taxonomischen Gruppen.

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1 General Introduction

1.1 Importance of drinking water

The availability of drinking water was one of the most important factors for the development of human settlements (70). With an increasing population the natural supply of water became limiting and the population was forced to develop new techniques to obtain access to other distant water reservoirs. High population densities have lead to increasing amounts of wastewater and contamination of the drinking water necessitating the development of sanitation and waste water treatment in the 19th and 20th century (61). Up to now, drinking water maintained its role as the most important consumer good worldwide. International guidelines for drinking water production and distribution were implemented intending to ensure high quality of drinking water for the majority of mankind (20, 76). The World Health Organisation (WHO) summarises the requirements for save drinking water as follows:

"The most common and widespread health risk associated with drinking-water is microbial contamination, the consequences of which mean that its control must always be of paramount importance. Priority needs to be given to improving and developing the drinking water supplies that represent the greatest public health risk." (76)

1.2 Drinking water in the EU and Germany

In Europe, the *Council Directive 98/83/EC on the quality of water intended for human consumption* regulates the drinking water quality for all members of the European Union (EU). Besides the quality of drinking water, the main objective of this directive is to protect "human health from the adverse effects of any contamination of water intended for human consumption by ensuring that it is wholesome and clean". It ensures that the drinking water "is free from any micro-organisms and parasites and from any substances which, in numbers or concentrations, constitute a potential danger to human health" (20).

In Germany the EU Council Directive is transferred into the national drinking water ordinance, the *Trinkwasserverordnung (TrinkwV 2001)* (13). It regulates the maximum contaminant levels of different adverse contaminants in drinking water to obtain a good quality of drinking water. The following conditions are governed:

1. Pathogenic microbial contaminants. These contaminants include protozoa, bacteria and viruses, whereas for viruses until now no maximum contaminant level is defined in the *Trinkwasserverordnung*. (For details of microbial pathogens see chapter 1.6)
2. Chemical contaminants. These contaminants include organic and halogen-organic contaminants such as polycyclic aromatic hydrocarbons (PAH) or trichloromethane and inorganic contaminants, heavy metals, and ions such as mercury or nitrite.
3. Physical and chemical parameters such as radiation (total indicative dose) or pH.
4. Sensory impairments such as flavour, odour or turbidity.

All conditions have to be fulfilled by the drinking water supplier in order to provide the population with clean and healthy drinking water. To obtain raw water, four major abstraction methods are used in Europe:

1. Abstraction from ground water. Groundwater is located beneath the ground surface and accumulates in aquifiers which are separated from each other by ground layers of low permeability. Mostly, groundwater is abstracted from confined aquifiers to avoid contamination from rain and surface water.
2. Abstraction from spring water. Spring water is ground water that flows to the ground surface from underground aquifiers.
3. Abstraction by bank filtration. In a distance to a water body, raw water is abstracted from the ground. Lake or river water passes through the soil of the bank, undergoing a physical and biological filtration.
4. Abstraction from surface water. In natural lakes or artificial reservoirs the raw water is abstracted mostly from the hypolimnion of the reservoir. But also river water abstraction occurs as well as the abstraction of artificial ground water. Artificial groundwater is prepurified surface water which is seeped and then abstracted from the ground.

After abstraction, the raw water undergoes several water treatment procedures, depending on the abstraction method and the quality of raw water. (For more details on water treatment see chapter 1.3). In Germany, a total of 5127×10^6 m³ of drinking water was produced in the year 2007. The vast majority of the raw water was abstracted from ground water (62%), followed by surface water abstraction (22%) (67). Surface water abstraction is especially prominent in German low mountain ranges such as the Harz Mountains and the Sauerland, where, due to geological conditions, artificial dams have

the ability to provide large fresh water reservoirs for nearby industrial regions with high population densities. Spring water abstraction and bank filtration both provide 8% of the total drinking water abstraction in Germany (*Figure 1.1*).

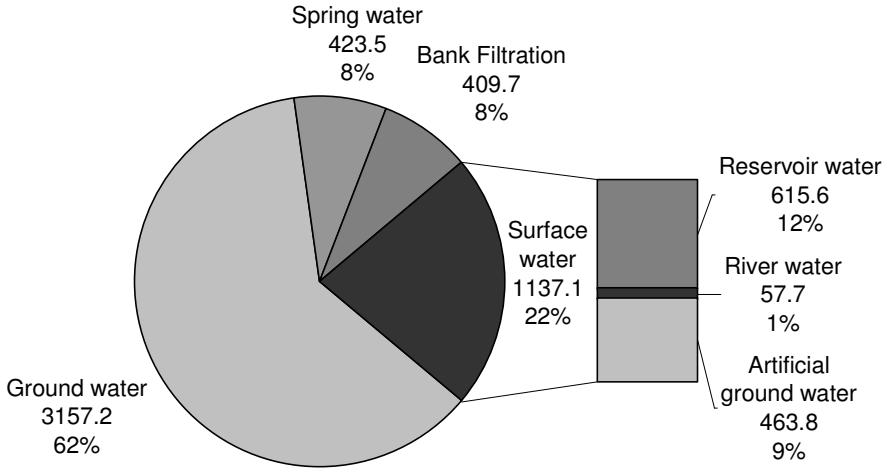

Figure 1.1: Different methods of drinking water abstraction in Germany 2007 (67). Data depicted in 10^6 m³.

1.3 Drinking water supply of the city of Braunschweig

For the present thesis the drinking water of Braunschweig was analysed. The city of Braunschweig is supported by the local water supplier *Harzwasserwerke GmbH*, which delivers the water to the border of the city, where it is transferred to the municipal distribution network. The water supply company Harzwasserwerke provides a total of two million people with drinking water and has an average output of about 80×10^6 m³ of drinking water per year. Braunschweig is supplied with drinking water stemming from two reservoirs, the Ecker reservoir and the Grane reservoir, both located in the northern part of the Harz mountain range about 40 km south of Braunschweig (19).

The Ecker reservoir is an artificial dam retaining the water from the Ecker (51° 50′ 8″ N, 10° 35′ 15″ E). It provides a maximum capacity of 13.3×10^6 m³ with a catchment area of about 19.0 km². Its low pH-value of 5.2 is explained by its dystrophic ecology leading to low amount of nutrients. The aerobic raw water is abstracted in the hypolimnion at a depth of 58 m.

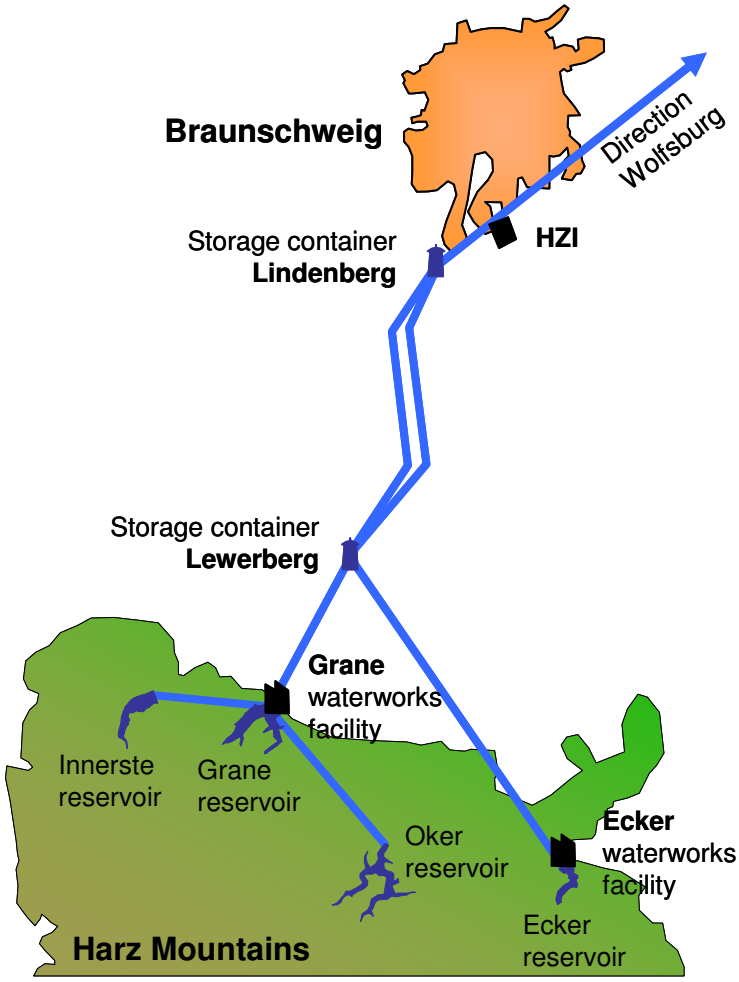

Figure 1.2: Relevant part of the water distribution network of the Harzwasserwerke GmbH.

The Grane reservoir (51° 54′ 32″ N, 10° 22′ 28″ E) is located in the north western part of the Harz Mountains and provides a reservoir volume of maximum 46.39 Mio. m³. The watershed of the Grane River alone, which is retained by an artificial dam, has a size of 22 km², but due to several pipelines to other rivers and reservoirs, the total catchment area of the Grane reservoir consists of 227 km². These pipelines include connections to the Oker River and the Gose River, as well as a pipeline to the Radau River and the Große Romke River. Additionally, there is a pipeline connection to the Innerste reservoir. Its oligotrophic water has a rather neutral mean pH-value of 7.2. Also in the Grane reservoir, the raw water is abstracted from the hypolimnion in a depth of 50 m. In the year 2003, 23% of the raw water stemmed from Ecker reservoir, while the majority of the raw water (77%) was abstracted from the Grane reservoir (19).

After abstraction from the hypolimnion of both reservoirs the raw water is treated in both waterworks by physical and chemical means. Pipe systems lead from both waterworks to the storage container Lewerberg, where both waters are mixed (*Figure 1.2*). From here, two pipelines transfer the water to the next storage container Lindenberg, which is located south of Braunschweig. The municipal drinking water supply (*BS Energy AG & Co KG*) is connected to this storage container. The Helmholtz Center for Infection Research (HZI) campus is directly attached to this municipal water supply. At the HZI the water undergoes again some treatment steps such as pressure reduction or filtering to remove tough grime, before it reaches the tap in the laboratory. In total, the flow time from the waterworks to the tap is approximately 36 h to 48 h.

The treatment of raw water (*Figure 1.3*) includes several treatment steps, five main steps are listed below (22):

1. Water hardening and pH adjustment (Ecker waterworks facility). Because of its dystrophic ecosystem, the raw water stemming from the Ecker reservoir is acidic and extremely soft, i.e. it has a very low concentration of carbonates. To prevent corrosion in the tubing system the raw water has to be hardened.
2. Flocculation. Suspended particles such as dissolved organic carbon (DOC) or microorganisms are aggregated by flocculating agents such as aluminium sulphate or iron (III) hydroxide. After flocculation, the turbidity and many bacteria are removed from the raw water.
3. Filtration. The water is filtered through grit and sand to remove remaining suspended particles and unsettled flocs.
4. Deacidification. To regulate the calcium carbonate-carbonic acid equilibrium after mixing the Ecker water with the water of the Grane reservoir and therefore again to prevent corrosion in the tubing system, the pre-treated water has to be hardened finally.
5. Chlorination. Disinfection of the pre-treated water is done by adding a different chlorine concentration, depending on the type of water, especially its pH and DOC concentration. For example, in the in the processed water stemming from the Ecker reservoir, the chlorine concentration is 0.6 - 0.7 mg/l and in the water stemming from the Grane reservoir, the chlorine concentration is 0.2 - 0.3 mg/l.

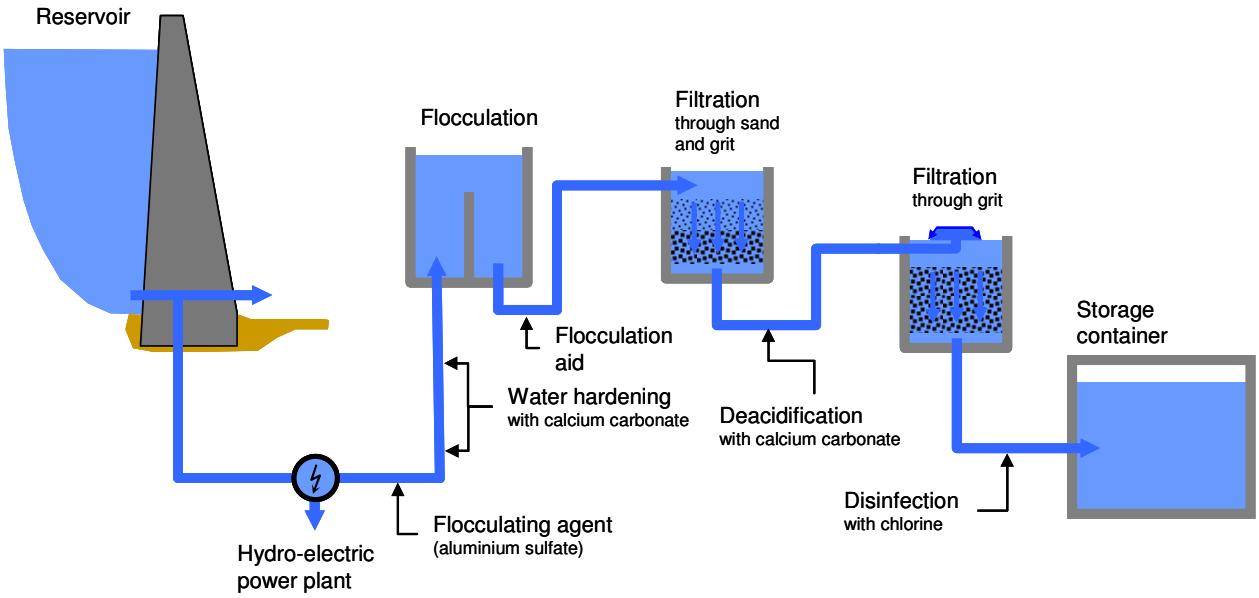

Figure 1.3: Water treatment at the Ecker waterworks facility (modified from (22)).

1.4 Bacteria in bulk water and drinking water biofilms

Although water treatment and disinfection measures aim at providing the customer with clean and pathogen free water, the water quality at the customer tap does not necessarily provide the same quality as the drinking water leaving the waterworks facility. Commonly in Europe drinking water is disinfected with chlorine and/or ozone addition to the processed water to kill any existing microbe. Although the vast majority of bacteria is killed by those potent oxidants, few bacteria are able to survive this procedure. In addition, several studies showed that chlorine and/or ozone used for disinfection reacted with complex organic matter in the drinking water converting it into higher levels of assimilable organic carbon (AOC) (39, 55). Due to elevated concentration of AOC the few surviving bacteria are able to regrow to a certain extent (19). In Braunschweig, typical abundances of bacteria in drinking water enumerated by epifluorescence microscopie ranged from $1x10^8$ to $5x10^8$ bacterial cells/l (chapter 3). According to Niquette et al, important factors influencing the regrowth of heterotrophic bacteria in a drinking water supply system are: 1) the concentration of organic compounds, 2) the chlorine concentration, 3) the residence time of the water in the distribution system, 4) the water temperature, and 5) the physicochemical characteristics of the material lining the distribution pipes (49).

Regrowth of bacteria may lead to several undesirable consequences for drinking water (35, 49, 73). Not only that bacterial regrowth may change the taste and the odour in an undesirable manner, the composition of regrown drinking water bacteria may promote the survival and growth of hygienically relevant or potentially pathogenic bacteria. In addition, the regrowth enhances the growth of protozoan grazers or predators of bacteria such as amoebae (35). Being the basis of a complex food chain, regrown bacteria can even support the reproduction of undesirable macroorganisms such as the crustacean *Aselus aquaticus* (38).

Regrowth does not only occur in bulk water, it also occurs in biofilms covering the surfaces of the tubing material (15, 59, 74). The interface between bulk water and the tube material is a prime site for the accumulation of bacteria and organic matter (8). Thus, biofilms are present in every drinking water supply system posing an issue of great relevance for public health. As many bacteria have the ability to attach to surfaces, biofilm formation occurs in many bacterial species (30). Biofilms in drinking water consist of many species all over the domain *bacteria* interacting in complex food webs (5). Various undesirable water-borne microorganisms are able to colonise biofilms, which may then be regarded as a reservoir for potential pathogens. Two possible mechanisms are proposed for the colonisation of biofilms (5): 1.) The microorganisms are not able to multiply in the biofilm habitat but they are able to survive there for several days to month, causing a transitory contamination (e.g. protozoa such as *Cryptosporidium*, *Giardia*; Bacteria such as *Pseudomonas*, *Helicobacter*, *Campylobacter* and some species of coliforms). 2.) The microorganisms are able to adapt and persist in the biofilm. Biofilms provide a multitude of different electrochemical and nutritive micro-environments enabling the potential pathogen to colonise it (e.g. *Mycobacterium spp.*, and *Legionella spp.*). Whether the contamination of the biofilm is transitory or stable depends on the competition between bacterial species, the availability of nutrients, or the genetic make-up of the bacteria within the biofilm (5, 28, 34). In addition, bacteria in drinking water are more protected against adverse environmental conditions such as disinfection measures (17, 58). The microbial aggregation state including the biofilm matrix molecules leads to a reduced diffusion of oxidants used for disinfection (68). Moreover, many bacteria colonising the drinking water biofilm developed sophisticated antioxidant strategies to evade oxidative stress (69).

As drinking water biofilms may harbor some bacteria that are relevant for health issues, the incorporation of biofilm should be avoided. Especially in the case of pressure loss events, the shear stress can disrupt pieces of biofilm. This causes not only an unpleasant colour and flavour of the bulk water but also a potential health risk (27, 37). A detailed analysis of the similarities and differences between bulk water and biofilm communities and their interaction could help to value the potential health risk of drinking water biofilms in a distribution system.

1.5 Bacteria in hot drinking water

Although hot drinking water is a frequently used consumer good, little is known about the community composition of the bacteria in hot drinking water supplies. Only few studies on domestic hot water system were done. In the early 1970s Brock et al. observed thermophilic bacteria with similarities to bacteria belonging to genus *Thermus* in laundry and domestic hot-water heaters, confirmed by later studies (2). Most of the studies on hot drinking water were concentrating on the human pathogens belonging to *Legionella* spp. (12, 54).

In other studies, it was shown, that the number of bacteria in hot water even may exceed the number of bacteria in the corresponding cold drinking water (11). Bagh et al. focussed on the investigation of total heterotrophic plate counts (HPCs) and total direct counts in hot drinking water compared with cold drinking water. Interestingly they found out that ratio between HPC and acridin-orange direct counts (AODC) in hot water was approximately 20 times higher than the ratio in cold drinking water. Therefore, it became clear that there were substantial differences in the community structure of cold and hot drinking water. However, there is a lack of studies about the total bacterial community structure and composition in hot drinking water.

1.6 Microbial pathogens in drinking water

The microbial of pathogens in drinking water are mentioned in the WHO guideline for drinking-water quality as the “most common and widespread health risk associated with drinking-water”. Thus, all disinfection measures aim at reducing the amount of pathogenic and potentially pathogenic bacteria for human health. Many drinking water pathogens or the diseases caused by them are well known to drinking water suppliers since the science of water microbiology developed. Well known drinking water

pathogens are for instance *Campylobacter jejuni*, toxigenic species of *Escherichia coli* or in Europe historical prevalent species such as *Vibrio cholerae*. Other bacteria were just recently recognised as potentially pathogenic or emerging in drinking water such as *Legionella* spp., *Pseudomonas aeruginosa*, or bacteria belonging to the *Mycobacterium avium* complex (64). These pathogens belong mainly to environmental bacteria introduced by pipe leakages or survival of the disinfection measures into the water distribution system where they find favourable environmental condition ensuring their growth and survival. In particular, biofilms or dead ends of the distribution system were transitory or stable contaminated, representing a permanent reservoir for those pathogens (see chapter 1.4).

There are a number of reasons for the emergence of "new" pathogens applying to different bacteria in a different manner. The most important reason is probably the development and application of new detection methods including molecular detection such as PCR, which was a major breakthrough in our ability to demonstrate the responsibility of agents as causes of waterborne diseases (see chapter 1.3.1). In addition, human lifestyle and behaviour changed dramatically within the last century. The globalisation of commerce and travel led to the introduction of hitherto unknown pathogens and the habit of showering and using air conditioning introduced new possible infection routes through aerosols (64). For instance, *L. pneumophila* was not discovered until the late seventies just because infections with *Legionella* were rare due to different showering behaviour and it could not be detected because it is not able to grow on standard agar media. The increase of elderly people in western countries due to improved medical care, patients receiving chemotherapy, and the emergence of AIDS led to increasing numbers of immunocompromised persons which are more susceptible to opportunistic pathogens in drinking water (51, 64). These persons can be subjected to infections which normally do not occur in healthy adults with a good immune status.

1.7 Cultivation dependent and cultivation independent detection methods

Up to now, the determination of heterotrophic plate counts (HPC) is recommended in most drinking water regulatories as the standard monitoring tool for a general microbial water quality assessment (4, 13, 20, 23, 76). HPC was invented originally by Robert Koch in 1881 and was one of the first techniques used for drinking water analysis (14). By HPC all organisms are recovered which can grow and form visible colonies on a complex nutrient-rich media. Although it has been shown in different studies, that the HPC greatly underestimate the actual numbers and the diversity of the bacterial microflora in drinking water, HPC is still considered to be a useful tool to obtain information about: 1) the efficiency of drinking water treatment processes, 2) changes in drinking water quality during distribution and storage, and 3) microbial regrowth (14). However, it was shown that the number of bacteria in aquatic environments directly counted using unspecific staining such as DAPI or SYBRgreen exceeded the number of bacteria determined by cultivation based methods such as HPC by several orders of magnitudes. This was called in literature the “great plate count anomaly” (66). The ratio of the bacterial abundance measured with cultivation based methods and the abundance measured with direct counting in aquatic environments is generally below 1%; often even lower (14). Several factors are currently considered to be responsible for this discrepancy:

1) Many bacteria exist in a viable-but-non-culturable (VBNC) state. In this state, metabolic activity of cells is typically low while they are unable to form visible colonies on culture media (28).
2) Most aquatic environments, especially drinking water, exhibit a low nutrient availability. In contrast, cultivation media contain very high substrate and nutrient concentrations. Only those bacteria that are adapted to those high concentrations are recovered by cultivation based methods. Some bacteria may even be killed by high substrate concentrations (3).
3) Many bacteria are slow growing or specialised to low temperatures (psychrophilic). By incubation at 22°C or 36°C according to the *Trinkwasserverordung*, they could be overgrown by fast growing mesophilic bacteria. Thus they were not detected on the plate after the incubation time (3).

4) Some bacteria, such as *Legionella spp.,* live intracellulary in protozoan hosts and have a complex life cycle that strongly affects their activity. Standard cultivation methods are not able to simulate this environment and cannot provide the right growth conditions.

Thus, cultivation based methods have considerable drawbacks for determining the structure and composition of aquatic environments. To avoid the bias of cultivation based detection methods, new molecular detection methods were developed in the 1980s (52). In addition, with the concept of a molecular taxonomy based on a comparative analysis of 16S rRNA a new era started (75). With the broader application of PCR in the 1990s a powerful tool became available for cultivation-independent detection. The PCR-technique allowed amplifying genes of desired specifity ranging from the strain-level to the phylum-level. The detection of a species specific gene meant therefore also the detection of the relevant species. Working with nucleic acids (NA) of relevant microorganisms no cultivation bias could affect the results, allowing also detecting viruses. In addition, a much higher sensitivity for most species was achieved, necessitating simply an upstream NA-extraction, the PCR reaction and a following electrophoresis for the visualisation. The disadvantage of this technique is that besides matching the detection limit, no information about the abundance of the respective bacterium is gained. Therefore, only presence/absence information was gained with simple PCR methods. This problem was solved with the development of the quantitative real-time PCR (q-PCR). In this method, the amplification of genes could be observed during the reaction process: The increase of the PCR-product was set into relation to defined standards and thereby a quantification of the initial template concentration was achieved. Nowadays a big variety of products using these principles are commercially available for the molecular diagnosis of different pathogens ranging from viruses (such as *Norovirus*) over bacteria (such as *Legionella pneumophila*) to protozoa (such as *Giardia*). However, sequence information about the relevant organism is compulsory. It makes this method alone inappropriate for the analysis of unknown environmental bacterial communities.

1.8 Community fingerprinting methods using 16S rRNA gene analysis

Nucleic acid based techniques have become the method of choice for determining the microbial community of natural environments and they proved to be an invaluable tool for characterisation of the structure of microbial communities. Particulary they have been used to determine the identities of microorganisms that have never been cultivated, and in some cases to predict their functional roles (56). Most approaches were focussing on the 16S rRNA gene of the small ribosomal subunit as a universal gene that every bacterium possesses. To provide a holistic view of natural microbial communities, a variety of 16S rRNA gene based methods have been developed.

One of the first widely adopted methods in microbial ecology was the denaturating gradient gel electrophoresis (DGGE) in the early 1990s (47). This method relies on the separation of PCR-amplified 16S rRNA genes using heterogenities in the GC-content of the amplified gene, leading to differences in their melting properties, although the amplicons all possess the same length. One of the primers carries a GC-rich clamp of around 40 bp. When running the gel, the amplicons are separated according their molecular weight. As the amplicon progress through the gel it is subjected to an increasingly higher chemical gradient of a denaturating compound. This leads to an increasing melting behaviour dependent on the GC-content and the sequence of the amplicon. In the end, the amplicon has a "butterfly-shaped" appeareance and its migration in the gel is strongly retarded compared to unmelted molecules (50). Whereas a chemical denaturation gradient is used for the DGGE, a temperature gradient is used for denaturation of the DNA in the temperature gradient gel electrophoresis (TGGE) (48, 50, 56). Another electrophoretic method is the single stranded conformation polymorphism (SSCP), which is widely used in mutation analysis, but has been adapted to the analysis of microbial communities (63). In contrast to DGGE and TGGE, SSCP is not based on double-stranded DNA, but on single-stranded DNA, which adopts a secondary / tertiary structure under non-denaturating conditions. To generate this single-stranded DNA, two general methods exist. Either, one strand is biotinylated using a biotin-labelled primer in the PCR and this strand is fished with streptavidin coated magnetic beads (19), or the other strand is phosphorylated and it is digested using lamda-exonuclease (63). The secondary structure of the ss-DNA is determined by intramolecular interactions, thus it is sequence dependent. The migration through the gel-matrix is affected by the secondary structure; it is hampered by spread out

molecules and fast with compact molecules (50). For complex environmental communities the result of a DGGE, TGGE or SSCP electrophoresis is a pattern of bands in a lane (fingerprint of the community), with each band ideally corresponding to one species / phylotype in the original community. The relative intensity of each band represents a quantitative measure for the relative abundance of this population in the analysed community. The bands can be excised, reamplified by PCR and subsequently sequenced to obtain information about the taxonomic position of the species. Additionally, phylogentic analyses can be conducted with the 16S rRNA-gene fragments up to the species level to estimate their functional role in the community (56). Another widely used approach commonly not based on gelelectrophoresis, but on capillar-electrophoresis is the terminal restriction length polymorphism (TRFLP). For this method, the 16S-rRNA is amplified with PCR, using a 5' attached fluorescent dye so that the amplicons become labelled. Amplicons are subsequently digested, using typically a 4-base restriction enzyme that cleaves at different positions in the gene, depending on the sequence (56). The mixture of restricted PCR-products is then loaded on a capillary normally used for sequencing with the ability to resolve to length dimorphisms of only a single nucleotide. While running, only labelled terminal fragments are detected by a laser detector. The outcome is an electropherogramm based solely on one parameter, the fragment length (50). This allows to compare the results with entries in ribosomal database, performing an *in silico* digestion with these entries before, and finally to get information about the phylogeny of the resulting restriction fragment. Additionally the relative abundance of each population can be estimated by dividing the peak area of the restriction fragment by total peak area (18). However, the specifity of the phylogenetic analysis is lower as in other fingerprinting techniques, because an individual terminal restriction fragment may correspond to 15 or more species (41). Moreover, incomplete restriction digestion may result in additional restriction fragments and therefore in an overestimation of the diversity. Recent developments in sequencing technology have led to another electrophoresis independent method for bacterial community analysis based on pyrosequencing (65). Pyrosequencing is based on sequencing by synthesis and relies on microscopic beads that attach to the PCR-products. These beads are then placed in a picotiter plate with 1.6 million wells and only one bead is distributed to each well. Then a mix of DNA-polymerase and an enzyme combination is added catalysing a light signal, when the DNA chain is prolonged. During the sequencing, the four nucleotides are washed

sequently over the bead. When the DNA-polymerase adds a nucleotide to DNA-strand, the resulting light signal, which is proportional to the number of incorporated nucleotides, is detected by a CCD-camera. In the end, a large dataset of 1.6 million sequences is produced, which has to be analysed by specialised bioinformatic software. The number of identical sequences divided by the total number of sequences is equivalent to the relative abundance of a phylotype. However, pyrosequencing is a sophisticated but expensive technique with the need of specialised scientist to work with. Further drawbacks are the relatively short sequences (appr. 80 bp) obtained from the first generation sequencers (65). Additionally, pyrosequencing results have to be analysed carefully, because transcription errors of the DNA-polymerase occur quite often, leading to a slightly differing sequence to the original sequence. Thus, pyrosequencing may result in an overestimation of the number of different species in an environmental sample. Major drawbacks of all molecular methods are the PCR-bias, meaning that the PCR-efficiency is not the same for each sequence, which could lead to biased relative abundances. Therefore the future might lie in the direct sequencing of environmental DNA (45).

1.9 Activity and viability of bacteria in natural communities

Only limited numbers of chromosomes are present in bacterial cells, therefore also limited numbers of 16S rRNA-gene are present. Targeting 16S rRNA-genes, DNA-based techniques can be used to assess information about the presence and the relative abundance of single phylotypes. This allows calculating community measures such as richness and other diversity indices. Unfortunately, high relative abundance does not necessarily mean that the relevant species is also active under the present conditions. Thus, DNA based fingerprint analyses cannot say much about the activity of a relevant species present in the environment. However, for the assessment of the relevance of a bacterium in the respective environment it is essential to gain information about its activity or viability, especially in the case of potential pathogens in drinking water. In contrast to 16S rRNA-gene concentration, the concentration of the ribosomal RNA of the small subunit, the 16S rRNA, is dependent on the ribosome content of the bacterial cell, which rises with increasing growth rate or activity. Thus, 16S rRNA based fingerprints can be seen to be a measure for bacterial activity (19, 26, 31, 40). In 1996, Teske *et al.* were the first to use this combined approach with DNA- and RNA based

fingerprints of bacterioplankton in a marine ecosystem (71). Further validation of this concept was done by a study showing that those bacteria detected by an RNA-based analysis were actively degrading ^{13}C-labelled pentachlorphenol (40). This leads to the assumption that the comparison of DNA based and RNA based 16S-rRNA community fingerprints can provide a line of reason for identifying the active members of the core community (10). Many studies showed that in aquatic ecosystems, including marine, freshwater and drinking water, a great discrepancy between RNA and DNA based fingerprints could be found (19, 46, 72). For the drinking water stemming from the Harzwasserwerke, it was shown that the discrepancy already found in the reservoir community also applies to the drinking water community (19). Especially in surface environments with high oxygen concentrations this effect is apparent, while in anoxic aquatic environments or in soils and sediment this discrepancy is not found (10). Brettar *et al.* hypothesised a potential role of oxidative stress as a mechanism to explain the differences in these environments.

Beside activity, viability is a crucial factor to determine the physiological status of a bacterium and is therefore important for quality and risk assessment in drinking water. In the last decade parameters that are linked to cell viability have been extensively investigated (29). Most common became the application of fluorescent dyes enabling the viability staining of environmental samples for *in situ* studies. A broad range of fluorescent dyes that can be used for the distinction of live and dead cells under the microscope is commercially available, aiming at different physiological targets in or at the cell, including membrane potential, enzymatic activity and membrane integrity (6). It is assumed in literature that membrane injured bacteria can be considered as dead, thus using the membrane integrity as a criterion for the distinction of live and dead cells seems to be valid for drinking water analysis. For this “live/dead”-staining procedure, two nucleic acid dyes are used, Propidium Iodide (PI) and SYTO9. SYTO9 is able to pass the intact membrane of all cells and binds to intracellular nucleic acids resulting in a green fluorescence (9). In contrast, PI is only able to enter the cell, when the cytoplasmic membrane is damaged. As a result of the “live/dead” staining, membrane intact cells appear as green, while membrane injured cells with the presence of both stains appear red (9).

1.10 The community composition of the drinking water microflora

At present, there are several studies that have investigated the composition of bacterial bulk water communities, mostly with molecular methods to identify the present bacteria at the phylum or class level (19, 57). In most studies gram-negative bacteria such as *Alpha*-, *Beta*- and *Gammaproteobacteria* and *Bacteroidetes,* were the most abundant bacteria, but also high numbers of gram-positive bacteria like *Actinobacteria* were found in bulk water of the drinking water supply system (DWSS). Only few studies identified bacteria up to the species level and included not only DNA-based but also RNA-based techniques (19, 57). The RNA-based analysis enabled to detect additional phyla such as *Planctomycetes*, *Cyanobacteria*, *Acidobacteria,* and *Nitrospira*. On the species level nitrifiers like *Nitrosospira briensis, Nitrosomonas urea,* and *Nitrospira moscoviensis* were commonly found in bulk water. Therefore, using RNA-based fingerprints enables to screen for active phylotypes or to detect low abundant but active phylotypes that are not detected by DNA-based techniques (7, 19).

1.11 Seasonal dynamics of bacterial communities in drinking water

The seasonal dynamics of freshwater bacteria in natural environment, such as lakes and rivers, have been studied to a large extent. (1, 16, 24, 25, 33). By contrast, studies on the seasonality of drinking water communities are quite rare and concentrated mainly on cultivation based techniques (43, 44) or on total bacterial biomass (1, 49). As the strong influence of the bacterial community in source water reservoirs on the bacterial community in drinking water is already described (49), it is of high interest if the influence of meteorological or environmental parameters on presence and activity described for freshwater habitats also applies to the drinking water community. Niquette et al. reported a major impact of water temperature above 15°C on the activity of suspended bacteria (49). But also a high impact of precipitation to the microbial community in drinking water reservoirs is described, especially after heavy rain events (33, 60). However, no long term studies of the cold and hot drinking water community dynamics using molecular detection methods including an assessment of the activity of single phylotypes are known until now.

1.12 Drinking water biofilms and biofilm successions

Many studies have focused on the examination of artificial drinking water biofilms in model systems (30, 36, 42). Assuming that only minor changes occur after a rather stable biofilm developed, most biofilm studies concentrated on short-term studies with biofilms grown for only a few months. However, Martiny et al. (42) showed that a stable community in a drinking water biofilm needed years to be established. They describe in their model DWSS a four phase succession of a drinking water biofilm: In the first 14 days, a biofilm is formed by bacteria recruited from the planktonic population in bulk water. In the second phase, during the first eight month, cell numbers increase and the biofilm community is dominated by members of the phylum *Nitrospira*. The third phase is dominated by a change to a distinct community and the disappearance of the dominating *Nitrospira* during two years. In their model DWSS, the last phase is reached after three years and the mature biofilm consisted of a mix of heterotrophic and autotrophic bacteria with a rather even community structure. This four phase model was developed for biofilm growing on stainless steel. However, for young biofilms it is reported that different tubing material of model DWSS, such as copper, PVC or stainless steel, may affect the number of cells, the morphology, and the bacterial composition (17, 32, 59, 62). It has been reported for various pathogens, such as *Legionella pneumophila*, *Mycobacterium* spp., and *Helicobacter* spp. that they were primarily associated with or grow in biofilms (7, 17, 21, 53). Therefore, drinking water biofilms can function as an important reservoir for pathogens and may provide a source of bulk water contamination by exchange of bacteria between biofilm and bulk water (34).

1.13 Objectives of the thesis

The overall objective of the present thesis was to get insights into the bacterial drinking water communities and their associated habitats such as drinking water biofilm and hot drinking water. Therefore, it is of relevance, which environmental factors are influencing the presence and activity of the members of these communities and what interactions exist between these communities.

The first objective was to analyse the seasonal variation and dynamics of the bacterial community in drinking water to understand meteorological and other environmental factors that are inducing shifts and changes in the presence and activity of these communities. The following questions were posed: Which seasonal variation of the drinking water in presence and activity can be observed? What is influencing the seasonal changes in the drinking water communities? To this end, the presence and the activity of the drinking water community was monitored over the timeframe of 18 month using 16S rRNA and 16S rRNA-gene based SSCP fingerprints followed by sequencing of major bands to quantify and identify members of the respective communities. The gained dataset was compared with meteorological data to identify induced changes in the communities.

The second objective was to determine the community composition of hot drinking water that was prepared of the cold drinking water. Parallel to the cold drinking water, the seasonal variation in hot drinking water was investigated. The main questions posed for this objective were: What does the community composition of hot drinking water look like? To which extend is the community composition of hot water dependent on the composition in cold water? Which seasonal variation of the hot drinking water can be observed in DNA and RNA based fingerprints? What is influencing the seasonal changes in the hot drinking water communities? Simultaneously to the monitoring of cold drinking water, the hot drinking water, centrally prepared at the campus of the HZI, was monitored, using the same molecular approach.

The third objective was to analyse the bacterial community structure and composition in drinking water biofilms. Questions of interest were: What is the effect of the surface material on the biofilm composition? How big is the similarity between biofilms of different sampling sites, but originating from the same drinking water? Which are the most active species in drinking water biofilms? Is there an exchange between the bulk water community and the biofilm community? To answer these

questions, biofilm was sampled from different pipe materials at different locations in a small scale network, which has been built more than 20 years ago at the campus of the HZI. It was assumed that the sampled biofilms had reached a mature state in this network. Bulk water was sampled at different sites in the HZI network and two other sites in the inner city of Braunschweig, to assess the community composition of the drinking water and the accompanying biofilm across the city. With these bulk water and biofilm samples single-strand conformation polymorphism (SSCP) fingerprinting based on extracted DNA and RNA was applied to the amplified 16S rRNA genes followed by sequencing of major bands to quantify and identify members of the respective communities. The resulting fingerprints and communities of bulk water and biofilms were compared to analyse the activity and interaction of both communities and to determine the effect of different tubing material.

The fourth objective was the assessment of live and dead bacterial taxa in drinking. The main questions to answer were: Which bacterial taxa are present and which of them are live or dead? What are the abundances and proportions of live and dead bacteria in drinking water? How is the activity determined using 16S rRNA based fingerprints linked to membrane integrity? A combination of cellular analyses followed by molecular analyses was used to achieve this objective. In detail, we performed a live/dead staining to the drinking water microflora to distinguish between live and dead bacteria. A subsequent Fluorescence Activated Cell Sorting (FACS) was used to separate the live and the dead fraction of the drinking water microflora from each other. The unsorted drinking water, the live fraction and the dead fraction were then analysed separately by SSCP fingerprinting and sequencing to determine the respective community composition. Using this approach, the phylotypes found in the live and in the dead fraction could be successfully assigned and their abundances could be compared with those of the unsorted drinking water.

1.14 References

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Chapter 2

2 Polyvalent Fingerprint Based Molecular Surveillance Methods for Drinking Water Supply Systems

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2.1 Abstract

Despite the relevance for public health, surveillance of drinking water supply systems (DWSS) in Europe is mainly achieved by cultivation based detection of indicator bacteria. The study presented here demonstrates the use of molecular analysis based on fingerprints of DNA extracted from drinking water bacteria as a valuable monitoring tool of DWSS and was exemplified for a DWWS in Northern Germany. The analysis of the bacterial community of drinking water was performed by a set of 16S rRNA gene based fingerprints, sequence analysis of relevant bands and phylogenetic assignment of the 16S rRNA sequences. We assessed the microflora of drinking water originating from two reservoirs in the Harz Mountains. The taxonomic composition of the bacterial communities from both reservoirs was very different at the species level reflecting the different limnological conditions. Detailed analysis of the seasonal community dynamics of the tap water revealed a significant influence of both source waters on the composition of the microflora and demonstrated the relevance of the raw water microflora for the drinking water reaching the consumer. According to our experience, molecular analysis based on fingerprints of different degrees of resolution can be considered as a valuable monitoring tool of DWSS.

2.2 Introduction

Despite the relevance for public health, surveillance of drinking water supply systems in Europe is mainly achieved by cultivation based detection of indicator bacteria. This approach bears the risk of neglecting viable but non-culturable (VNBC) bacteria on the one hand, on the other hand, many pathogenic bacteria, including emerging ones are not monitored (7, 15, 22). Careful estimates indicate that each year about 350 million people are infected by waterborne pathogens with 10-20 millions succumbing to severe cases (20). This phenomenon is far from being restricted to developing countries but also threatens developed countries. In the USA almost 430,000 cases were reported in 126 outbreaks of waterborne infectious diseases from 1991 to 1999 (1).

Production of drinking water complying with international quality standards does not necessarily ensure good drinking water for the consumer (2). Re-growth of bacteria in the distribution system is a major problem that may have adverse effects on drinking water quality and is correlated with biofilm formation. The effects of re-growth may

range from effects on taste and odour to true health threats when it comes to re-growth of pathogenic bacteria (20). Key factors influencing re-growth of bacteria in a drinking water supply system (DWSS) are: i) concentration of organic compounds, ii) chlorine concentration, iii) residence time of the water in the distribution system, iv) water temperature and v) physico-chemical characteristics of the material lining the distribution pipes (13).

The bacterial community of drinking water plays a crucial role for the drinking water quality. It is the main consumer of the organic carbon in the drinking water, mineralizes it to CO_2 or other degradation products, nitrifies ammonium to nitrite and nitrate, and forms biofilms. The autochthonous microflora can sustain the growth of protozoa and metazoa (e.g. crustacean) that are visible to the consumer (4, 19) or may have adverse effects on the taste and safety of the drinking water (11). The microbial community of the drinking water may directly interfere with pathogenic bacteria, i.e. it can suppress or promote the survival and growth of hygienically relevant and potentially pathogenic bacteria (10). E.g. the formation of biofilms enables survival or even growth of pathogenic bacteria, while the competition for the same carbon sources or the production of antibiotic substances may suppress pathogenic bacteria. Since the microbial community is a key factor of drinking water quality with respect to many aspects, its analysis is a focus of our study.

The HEALTHY-WATER project, a project in the 6th Framework of the EU (http://www.hzi-helmholtz.de/en/healthy_water/) is aiming towards the development of new molecular detection technologies of microbial pathogens in drinking water with special emphasis on emerging pathogens (14). Among several approaches that are under development, fingerprint based methods and their results will be presented here, those especially have the potential to monitor the whole bacterial community and thus bear the potential to detect also unexpected pathogenic bacteria.

2.3 Material and Methods

2.3.1 Study site

The overall study comprises samples from a DWSS in Northern Germany that provides about 80 Mio m^3 of drinking water per year and is providing drinking water for about two million people. Source water of the DWSS is provided by two surface water reservoirs, an oligotrophic reservoir (Grane, pH 7.2) and a dystrophic reservoir (Ecker, pH 5.2). The collection of aerobic raw water is done from the deep water (50-58 m). More details on the DWWS are given by Eichler et al. (3). The focus of this study is on tap water and the seasonal changes studied from autumn 2006 to spring 2008.

2.3.2 Molecular methodology

The bacterial community of the water were harvested by filtering several liters of water onto a sandwich of a glass fiber GF/F plus 0.2 µm nuclepore filter (Whatman) (for details on the molecular methods see Eichler et al. (3)). In brief: DNA was extracted and purified; bacterial 16S rRNA gene amplicons generated by PCR were subjected to separation by non-denaturing acrylamide gel electrophoresis enabling Single Strand Conformational Polymorphism (SSCP) analysis. DNA based SSCP analyses were performed to follow the seasonal dynamics (5, 6, 18). The banding patterns on the SSCP gels, used as a direct measure of the community structure, were compared by cluster analysis (GelCompare II, Applied Maths). The composition of the bacterial community was determined by sequencing the single bands of the gel pattern and identifying the sequences by phylogenetic analysis using the international 16S rRNA gene sequence data base.

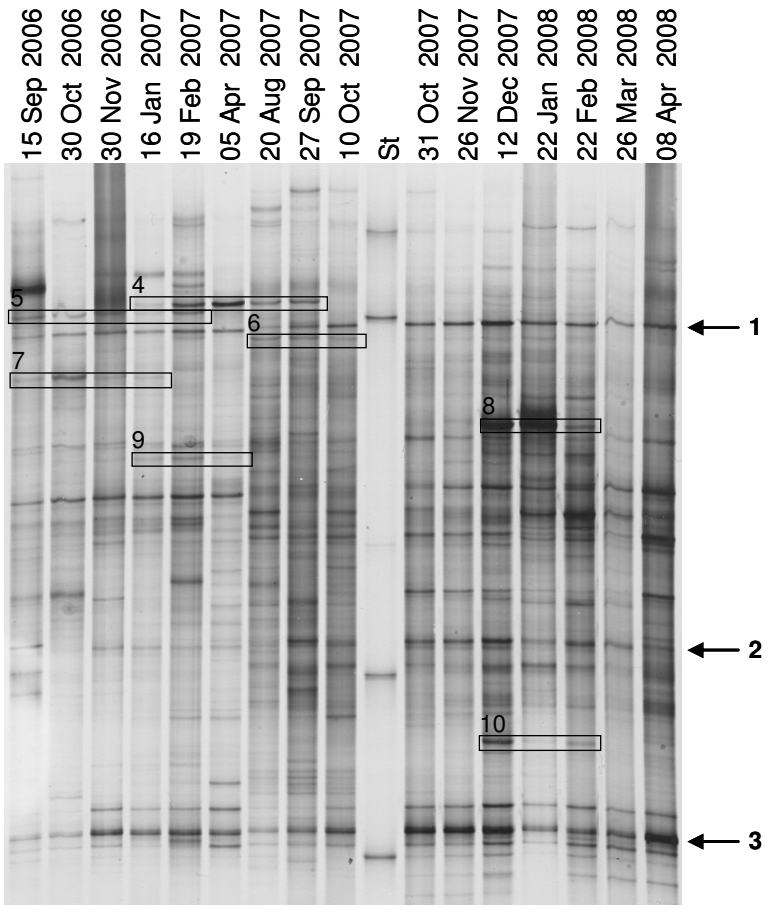

Figure 2.1: DNA based community fingerprints from tap water samples obtained at the indicated dates. Arrows indicate bands observed in all samples, bands in boxes are only observed during certain times of the year. St= standards of reference bacterial species.

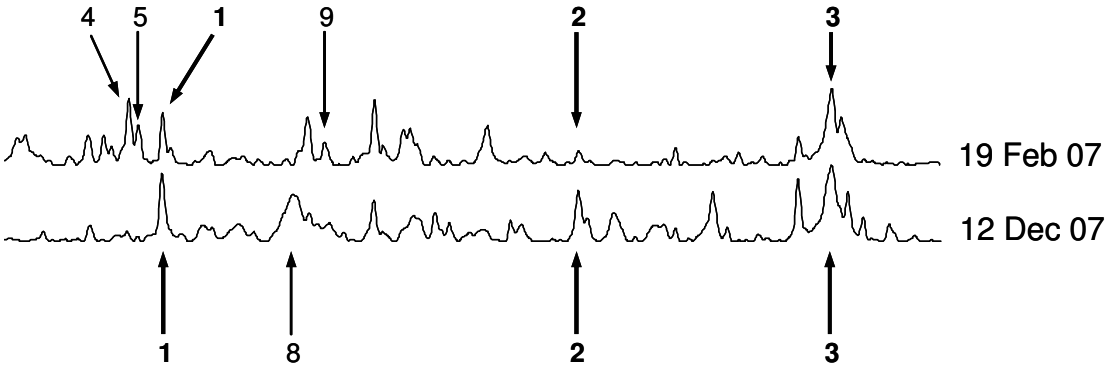

Figure 2.2: Density curves from the banding pattern of the community fingerprints from two different samples (constant bands 1-3 in bold). Band numbering is consistent with Figure 1.

2.4 Results and Discussion

2.4.1 Overall community structure of the drinking water microflora

The overall community structure of the drinking water microflora of tap water was assessed during one and a half years at monthly intervals to understand seasonal dynamics (Figure 2.1). These DNA based community fingerprints are banding patterns of single 16S rRNA genes separated according to sequence differences using SSCP electrophoresis. Ideally, the single bands represent different bacterial taxa at about the species level (17). For a detailed analysis of the single banding patterns density curves were produced using an electronic scanner (Figure 2.2). These density patterns show peaks, corresponding to the specific bands, and allow quantification of the amount of single strand DNA present in the single bands by integrating the area under the specific peak. A first comparison of the fingerprints shows that there are three major bands (1-3 marked with arrows in Figure 2.1) that occurred in all samples whereas several bands occurred only during certain times of the year (boxes 4-10 in Figure 2.1). The banding patterns of the single drinking water communities comprise about 40 to 80 different bands above the relative abundance threshold of 0.1% of the total DNA per lane. The constant bands represent 6-24% of the total DNA per lane leaving about 59-87% of the DNA for the variable bands. A seasonal pattern of the three constant bands can be recognized by comparing their relative amounts (Figure 2.3). Especially the most abundant band 3 shows a strong increase, from 3.6 to 16%, in October and a decline in January to March in both winters studied. Overall, these constant bands can be assumed to represent three different bacterial species that showed seasonal changes in their relative abundances by a factor of four according to the DNA abundance of the band. For a detailed understanding of the variation in the banding patterns, i. e. the community structure of the whole bacterial drinking water microflora, a cluster analysis was performed that allows a statistical comparison of the banding patterns of the different lanes (Figure 2.4). The cluster analysis revealed that the banding patterns changed in about 3 to 4 month intervals as revealed by the six main clusters in Figure 2.4. In addition, the cluster late summer 07 forms a subgroup with cluster autumn 07 as well as cluster winter 08 with spring 08. This sub-grouping indicates that the bacterial microflora is continuously changing, but mostly still related to the previous microflora.

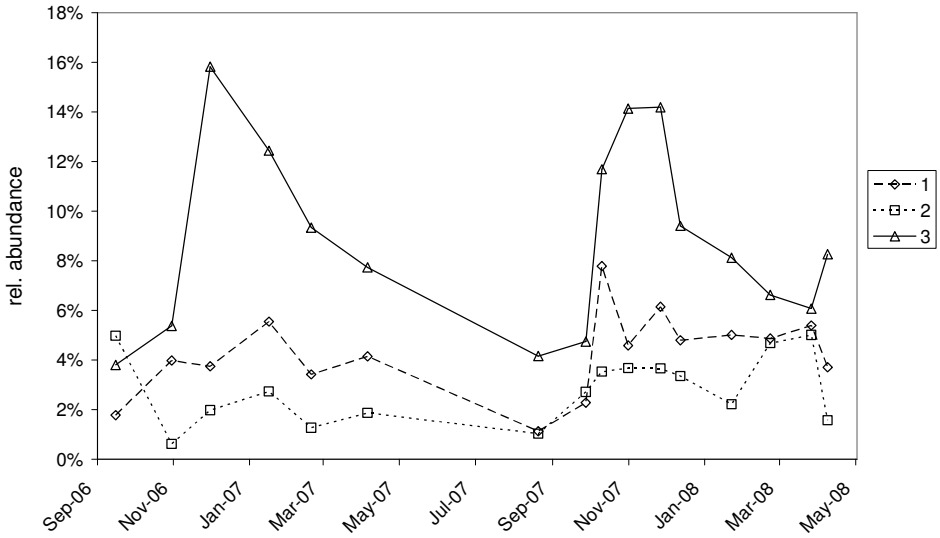

Figure 2.3: Seasonal variation of the relative abundances of the single strand DNA of the three major bands (1-3) representing three different bacterial species.

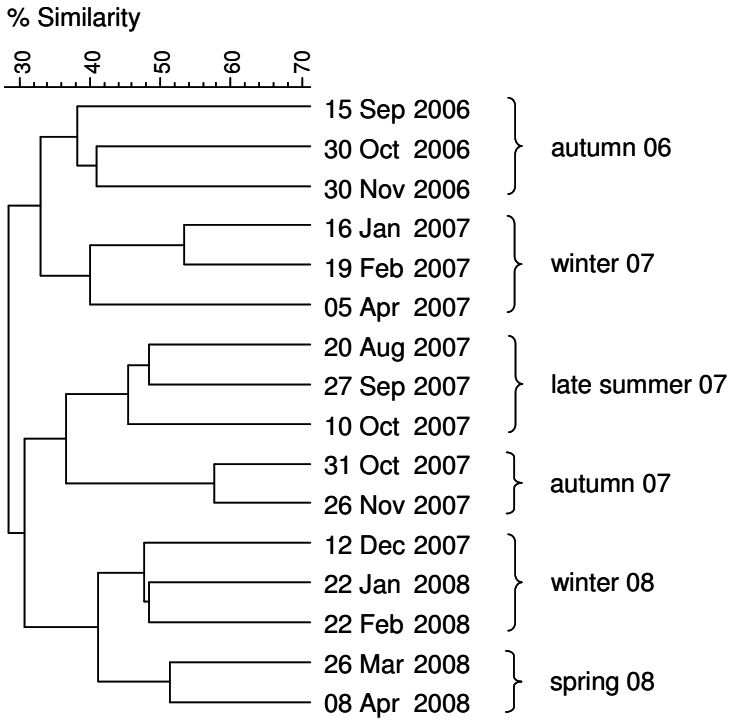

Figure 2.4: Cluster analysis of all banding patterns from the community fingerprints shown in Figure 1 (analysis was done by using GelCompare II (Applied Maths), Algorithms: Dice, Complete Linkage, all bands above 0.1% abundance included in analysis).

2.4.2 Taxonomic composition of the drinking water microflora

For identification of the single bacterial taxa represented by the bands of the community fingerprints, these bands have to be excised and sequenced. The generated 16S rRNA partial sequences (about 420nt) can then be compared with the large data set of bacterial 16S rRNA sequences available in international databases to identify the closest known bacterial species. In a previous study of the same DWSS, we identified 71 unique phylotypes, i. e. 16S rRNA gene sequences with a sequence similarity of > 98% and phylogenetic uniqueness as discrimination criteria that comprised most of the bacterial species in this drinking water community (3). Using these phylotypes as a reference data base, we could identify the three constant bands as the following bacterial taxa: band 1 = *Methylophilus* sp. (identical to phylotype 1 from Eichler et al. 2006, class *Betaproteobacteria*); band 2 = identical to phylotype 21 from Eichler et al. 2006, phylum *Actinobacteria*); band 3 = identical to phylotype 22 from Eichler et al. 2006, class *Alphaproteobacteria*). All three phylotypes belonged to bacterial species that have not been cultured and could only be identified by molecular analysis of DNA extracted from drinking water. In addition, all three phylotypes belonged to different bacterial classes or phyla indicating a large phylogenetic diversity of the drinking water microflora (23). As pointed out above, several bands (number 4 to 10) occurred only during a specific period and can be seen as indicators of changes in the structure and composition of the drinking water microflora. Sequence comparison of band 8 revealed that it was identical with phylotype 6 from Eichler et al. representing a *Betaproteobacterium* from the genus *Simonsiella*. This phylotype had only been observed before in the dystrophic Ecker reservoir microflora and can therefore be considered as an indicator for this microflora.

The analysis of the bacterial community by SSCP fingerprints has already been shown to be of great use for the study of the impact of the source water and the water treatment processes on the drinking water bacterial community. Eichler et al. have shown that the bacterial community structure of the raw water samples from the two reservoirs was very different reflecting the different limnological conditions of the reservoirs (highly dystrophic vs oligotrophic reservoir). No major changes of the structure of the bacterial community were observed after flocculation and sand filtration, while chlorination of the processed raw water strongly affected bacterial community structure as best reflected by the RNA-based fingerprints. According to assessment of the community composition by sequencing of abundant bands and

phylogenetic analysis of the sequences obtained, the taxonomic composition of the bacterial communities from both reservoirs was very different. After chlorination, growth of nitrifying bacteria was observed. Detailed analysis of the community dynamics of the whole DWSS revealed a significant influence of both source waters on the composition of the microflora and demonstrated the relevance of the raw water microflora for the drinking water provided to the end user.

2.4.3 Conclusions

- The DNA based community fingerprints allowed to follow the seasonal dynamics of the whole bacterial microflora in tap water.
- The SSCP fingerprints enabled the assessment of the relative abundance of all bacterial members of the drinking water microflora to a threshold of 0.1% relative abundance and, after sequencing, their taxonomic identification to the species level.
- The seasonal dynamics of the tap water microflora was characterized by three constant and 40-80 varying members of the bacterial community.

These insights into the bacterial community dynamics of a drinking water supply system obtained during this and the former study led us to recommend molecular analysis based on fingerprints of different degrees of resolution as a valuable monitoring tool of DWSS. The rapid overview gained on the DWSS bacterial community can be furthermore improved and accelerated by standardized formats of the molecular analysis.

2.4.4 Future perspectives and applications of fingerprints as tools for drinking water research and monitoring

In the future, SSCP analysis can be used to focus on specific pathogenic bacterial groups of interest what is currently under development in the Healthy-Water project. To achieve this goal, primers with a different degree of specificity are designed and applied to generate fingerprints for pathogenic bacterial genera or species of interest such as

Campylobacter, Arcobacter and *Helicobacter* (12, 16). Especially, with respect to biofilms, analyses of DWSS for these genera are of high relevance to human health (9, 22).

In many cases a higher phylogenetic resolution is needed than the one retrievable from the fingerprint band sequences in order to get a more precise taxonomic position of the target pathogenic bacterium. An improvement of the phylogenetic resolution can be achieved by designing highly specific primers and probes of a different degree of specificity based on the sequence of bands of interest. Using these highly specific primers allows the generation of a complete 16S rRNA gene sequence (>1400 nucleotides) of aquatic bacteria (8). This full 16S rRNA sequence allows a more precise analysis of the phylogenetic affiliation compared to the fragments obtained from the SSCP gel (about 420 nucleotides). Additionally, quantification of specific (pathogenic) bacteria by real-time PCR can be linked to SSCP-fingerprints. The above mentioned primers designed based on the fingerprint band sequences can be used for real-time PCR. This is of specific relevance when a new organism is detected by fingerprints that are of interest, e.g. suspicious to be a pathogenic or noxious bacterium, but not yet cultivated and the 16S rRNA sequence is not yet available in public data bases. These examples demonstrate the great potential of molecular fingerprint analyses for an improved monitoring of DWSS and a better understanding of possible hygienic risks related to various treatment and management procedures.

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Chapter 3

3 Seasonal dynamics of the bacterial community structure and composition in cold and hot drinking water

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3.1 Abstract

In the present study we investigated the bacterial dynamics in the drinking water of Braunschweig, Germany, originating from two reservoirs over the timeframe of 18 month. Using single stranded conformation polymorphism (SSCP) fingerprints of 16S rRNA and 16S rRNA-genes and sequencing major bands we analysed the seasonal dynamics of the community composition and its activity of cold and the corresponding hot drinking water sampled at the campus of the Helmholtz Centre for Infection Research (HZI) in relation to meteorological data of the catchment area. For cold drinking water it was demonstrated that the precipitation in the catchment area was the major impact influencing the amount of bacteria. The activity of the cold drinking water community was also mainly influenced by months of high precipitation, characterized by phases of high activity of only a single phylotype. For the hot drinking water the amount of bacteria was only 20% lower than that of cold drinking water bacteria. Its community was less rich than cold drinking water community and mainly composed of thermophilic and thermotolerant bacteria. The hot drinking water community composition was not influenced by environmental factors like precipitation or temperature. In contrast to cold drinking water, highly abundant bacteria in hot drinking water were also highly active.

3.2 Introduction

Three main types of drinking water production are generally distinguished: drinking water abstraction by ground water, drinking water abstraction by bank filtration, and drinking water abstraction by surface water. In the present study we concentrated on the investigation of the bacterial dynamics in the drinking water of Braunschweig, supplied by the Harzwasserwerke. This drinking water originates from two reservoirs, the Grane reservoir and the Ecker reservoir, where raw water is abstracted from the hypolimnion as described elsewhere in more detail (14). Treatment of the raw water included several treatment steps: 1. pH adjustment, 2. flocculation, 3. filtration, 4. deacidification, and 5. chlorination. Despite all treatment procedures complying with international quality standards, the drinking water at the consumer's tap does not necessarily provide the same quality as it leaves the treatment plant. Due to regrowth several problems ranging from effects on taste and flavor of the drinking water to the occurence of pathogenic bacteria may occur (28, 35, 42). Regrowth of bacteria in drinking water is mainly influenced by the concentration of organic and inorganic nutrients, chlorine, residence time of the drinking water in the distribution system, and temperature (35).

Bacterial communities in drinking water have been studied (27), mostly with molecular methods to identify the bacteria at the phylum or class level (6, 14). In these studies, the most abundant bacteria belonged to gram-negative bacteria such as *Alpha*-, *Beta*- and *Gammaproteobacteria* and *Bacteroidetes*, but also high numbers of gram-positive bacteria like *Actinobacteria* were observed. Only few studies included not only DNA-based but also RNA-based techniques (14, 38). The use of RNA-based techniques enabled to detect additional phyla such as *Planctomycetes*, *Cyanobacteria*, *Acidobacteria,* and *Nitrospira*.

All regulator guidelines for controlling the quality of drinking water in Europe include cultivation based methods such as heterotrophic plate counts and coliform counts as standards for the detection of bacteria (9). The problem of cultivation based methods that detect only the few bacteria growing under the respective cultivation conditions and non-culturability of viable-but-non-culturable (VBNC) bacteria, i.e. even under adequate cultivation conditions these bacteria do not grow due to physiological constraints (7), necessitates molecular detection methods, such as 16S rRNA-based and 16S rRNA gene-based fingerprints to analyse the bacterial community composition unbiased by cultivation drawbacks (10). Comparison of rRNA-based and 16S rRNA

gene-based fingerprints allows to gain insights into the activity of single phylotypes (21).

Although hot drinking water is a frequently used consumer good, little is known about the general community composition in hot drinking water supplies. Only few studies on domestic hot water system were done, and most of them were concentrating on potential human pathogens such as *Legionella* (24, 30). In the early 1970s Brock et al. observed thermophilic bacteria with similarities to bacteria belonging to genus T*hermus* in laundry and domestic hot-water heaters, confirmed by later studies (8, 36). In other studies, it was shown, that the number of bacteria in hot water even may exceed the number of bacteria in the corresponding cold drinking water (4). Bagh et al. focussed on the investigation of total HPC and total direct counts in hot drinking water compared with cold drinking water. Interestingly they found out that ratio between heterotrophic plate count (HPC) and acridin-orange direct counts (AODC) in hot water was approximately 20 times higher than the ratio in cold drinking water. Therefore, it became clear that there were substantial differences in the community structure of cold and hot drinking water. However, there is a lack of studies about the hot drinking water community structure and composition.

The dynamics of freshwater habitats have been studied several times (2, 11, 19, 20, 25). By contrast, studies on the seasonality of drinking water communities are quite rare and concentrated mainly on cultivation based techniques (31, 32) or on total bacterial biomass (2, 35). As the strong influence of the bacterial community in source water reservoirs on the bacterial community in drinking water is already described (14), it is of high interest if the influence of meteorological or environmental parameters on presence and activity described for freshwater habitats also applies to the drinking water community. Niquette et al. reported a major impact of water temperature above 15°C on the activity of suspended bacteria (35). But also a high impact of precipitation to the microbial community in drinking water reservoirs is described, especially after heavy rain events (25, 39). However, no long term studies of the cold and hot drinking water community dynamics using molecular detection methods including an assessment of the activity of single phylotypes are known to the authors until now.

The present study aims at 1) understanding the influence of meteorological or environmental parameters, such as temperature and precipitation, on the bacterial drinking water microflora, 2) the examination of the differences between the hot water bacterial community and the cold water community, and 3) the examination of the activity of the hot and cold drinking water microflora over the timeframe of 18 months.

3.3 Material and Methods

3.3.1 Study sites and sampling.

Cold drinking water was sampled monthly from in May 2008 to in October 2009. It was taken from the tap of laboratory D0.04 on the campus of the Helmholtz Centre for Infection Research (HZI), Braunschweig-Stöckheim, Germany, with five minute flushing to prevent stagnant water to be sampled. This drinking water originates from two surface water reservoirs (Grane reservoir, oligotrophic water, 51° 45' 44" N, 10° 22' 38" E), and Ecker reservoir, dystrophic water, 51° 50' 27" N, 10° 34' 45" E) situated in the Harz Mountains 40 km south of Braunschweig. Processing of the drinking water by the local supplier Harzwasserwerke GmbH included flocculation/coagulation, sand filtration and chlorination (0.2 - 0.7 mg l^{-1}). More details on the respective drinking water supply system are given elsewhere (14). Hot drinking water was sampled in May 2008, and regular monthly sampling was done from September 2008 to October 2009. It was taken from a shower next to lab D0.04 also with several minutes flushing to avoid the sampling of stagnated water. The hot drinking water was made from normal cold drinking water directly on the campus where it was heated to 60°C and transferred in insulated pipes to the shower. The hot water at the HZI campus was transported in a circular supply system.

Drinking water microorganisms from cold and hot drinking water were sampled by filtration according to Eichler et al. (14). In brief, 5 liters of drinking water were filtered through a filter sandwich consisting of a 0.2 µm pore size polycarbonate filter (90 mm diameter; Nucleopore; Whatman, Maidstone, United Kingdom) with a precombusted glass fiber filter on top (90 mm diameter; GF/F; Whatman). Biomass harvested on filter sandwiches was stored at -70°C until further analysis. In parallel direct counts were performed and relevant drinking water parameters, such as pH, conductivity, temperature, and chlorine concentration were determined. The monthly mean temperature of the cold drinking water, measured directly after sampling in Braunschweig is shown in Figure 1 a. The maximum temperature was 14.4°C in July 2008 and 16.9°C in August 2009, while the lowest temperature of 7.6°C was measured in January 2009.

3.3.2 Direct counts of drinking water bacteria.

For total bacterial counts, formaldehyde-fixed samples (2% final concentration) were stained with Sybr Green I dye (1:10000 final dilution; Molecular Probes, Invitrogen) for 15 min at room temperature in the dark. Five ml were filtered onto 0.2 µm pore size Anodisc filters (Whatman) and mounted with Citifluor on microscopic glass slides according to Weinbauer et al. (45). Slides were either analysed directly with epifluorescence microscopy or stored frozen (-20°C) until examination. For epifluorescence microscopy, a microscope (Axioplan, Zeiss) with suitable fluorescence filters was used and the slides were examined using 100fold magnification. For each sample, 10 micrographs were taken and image sections of defined size (0.642mm x 0.483mm) were analysed using the Image J software with the plug-in collection from MacBiophotonics (http://www.macbiophotonics.ca/). Typically, 500-800 bacterial cells per image were counted.

3.3.3 Nucleic acid extraction from drinking water

Cold and hot water DNA and RNA were extracted from the filter sandwiches. For extraction of DNA and RNA, a modified DNeasy/RNeasy protocol (Qiagen, Hilden, Germany) was used. In this procedure, sandwich filters were cut into pieces, incubated with lysis buffer containing 10mg/ml lysozyme (Sigma) for 60 min at 37°C (DNA) or 20 min at 20°C (RNA). After a proteinase K digestion (DNA) according to the manufacturer's instructions, the samples were heated to 70°C in a water bath for 20 min (DNA) or 15 min (RNA). After filtration through a polyamide mesh with 250 µm mesh size, absolute ethanol was added to the filtrate (ratio filtrate/ethanol 2:1) and the mixture was applied to the adequate spin-column of the Qiagen kit. From now on, the washing and elution protocol was followed according to the manufacturer's instructions.

For drinking water RNA, a subsequent on-column DNase digestion (RNase-Free DNase Set, Qiagen, Hilden, Germany) was applied. Nucleic acids were eluted from the columns with DNase/RNase free water and stored at -20°C. The nucleic acids were quantified using Ribogreen (RNA or ssDNA quantification, Molecular Probes; Invitrogen) or Picogreen (dsDNA quantification, Molecular Probes; Invitrogen) according to Weinbauer and Höfle (33).

3.3.4 16S rRNA and 16S rRNA gene based community fingerprints.

PCR amplification of 16S rRNA genes from the extracted nucleic acids were performed using the previously described primers COM1F (5´-CAGCAGCCGCGGTAATAC-3´) and COM2R (5´-CCGTCAATTCCTTTGAGTTT-3´), amplifying positions 519 to 926 of the *Escherichia coli* numbering of the 16S rRNA gene (40). For single strand separation a 5´-biotin-labeled forward primer was used according to Eichler et al. (14). From 16S rRNA, reverse transcription was carried out before PCR using the First strand cDNA synthesis Kit (Fermentas, Canada) following the manufacturer's instructions with the same COM-primers. PCR was carried out using 2 ng DNA/cDNA template in a final volume of 50 µl, starting with an initial denaturation for 15 min at 95°C. A total of 30 cycles (30s at 95°C, 30s at 55°C, and 1 min at 72°C) was followed by a final elongation for 10 min at 72°C. Amplification was achieved using HotStarTaq DNA polymerase (Qiagen, Hilden, Germany).

For the preparation of ssDNA and community fingerprints, the protocol described by Eichler et al. (14) was slightly modified. Briefly, magnetic streptavidin coated beads (Promega, Madison, Wis.) were applied to obtain ssDNA from the PCR amplicons. Quantification of the obtained ssDNA was performed on a 1.5% agarose gel by comparison with a low-molecular-weight marker (Invitrogen low-DNA-mass ladder). For SSCP fingerprinting analysis, 25 ng of the obtained ssDNA was mixed with gel loading buffer (95% formamide, 10 mM NaOH, 0.25% bromphenol blue, 0.25% xylene cyanol) in a final volume of 7 µl. After incubation for 3 min at 95°C, the ssDNA samples were cooled on ice, loaded onto a nondenaturing polyacrylamide-like gel (0.6x MDE gel solution; Cambrex BioScience, Rockland, Maine) and electrophoretically separated at 20°C at 400 V for 18 h on a Macrophor sequencing apparatus (Pharmacia Biotech, Germany). The gel was silver stained according to the method described by Bassam et al. (5). Dried SSCP gels were digitized using an Epson Expression 1600 Pro scanner, bands with an intensity of >0.1% of the total lane were considered for further statistical analysis. Similarity coefficients were calculated using Dice algorithm. Dendrograms were constructed with the Neighbor-Joining algorithm using the GelCompare II software (Applied Maths, Kortrijk, Belgium). Community indices were calculated using the software Primer 6 (PRIMER-E Ltd, Ivybridge, UK).

Sequence information from the single bands of the SSCP fingerprints was obtained following the protocol of Eichler et al.(14). Briefly, ssDNA bands were excised from the SSCP acrylamide gels, and boiled in Extraction buffer (10 mM Tris-HCl, 5 mM

MgCl2, 5 mM KCl, 0.1% Triton X-100, pH 9). 7µl of the extraction solution was used in a reamplification PCR with the unbiotinylated COM primers described above. These amplicons were purified (MinElute kit, Qiagen, Hilden, Germany) and subsequently sequenced by cycle sequencing (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit; Applied Biosystems, Foster City, Calif.). Before analysis on an ABI Prism 3100 Genetic Analyzer, the products were purified using the BigDye Terminator purification kit (QIAGEN). Phylogenetic identification of the sequences was done either by the NCBI Tool BLAST/blastn (1) for comparison with the closest 16S rRNA gene sequence and for the identification of the closest described relative or the Ribosomal Data Base Project Seqmatch and Classifier tool (12, 44) for determination of corresponding taxonomic groups (RDP Release 10, Update 18, Jan 25, 2010). When more than two definite base pair differences existed in comparison with other phylotypes, we defined a new phylotype.

3.3.5 Meteorological Data

The meteorological data of Clausthal-Zellerfeld was measured by the Institute for Electrical Information Technology at the Clausthal University of Technology (Figure 3.1 a). Data of Clausthal-Zellerfeld was chosen, because it is located in the middle of the West Harz representing best the catchment area of the Grane reservoir and the Ecker (14). The maximal monthly mean aerial temperatures in Clausthal-Zellerfeld were observed in August 2008 / 2009 with 16.0°C, while the minimum was observed in January 2009 with -1.8°C. Months of high precipitation were June / August 2008, February / March 2009, June 2009 and October 2009 with a volume of 100 l/m² and above.

3.4 Results and Discussion

3.4.1 General characterisation of the drinking water

Cold drinking water was sampled monthly beginning in May 2008, ending in October 2009. The mean temperature for cold water was 12.5°C and varied, depending on the season, between 7.6°C in January and 16.9°C in September (Figure 3.1 a). The pH of the cold drinking water varied between 8.2 and 8.8 with a mean of 8.5. During sampling period no chlorine was detected (detection limit of 0.1 mg/l) and measurements of conductivity during the last six months of sampling resulted in a mean conductivity of 142.8 ± 7.5 µS/cm. The number of total bacterial counts varied strongly between $0.78x10^8$ and $3.82x10^8$ cells/l with a mean of $2.41x10^8$ cells/l (Figure 3.1 b).

In hot drinking water the temperature fluctuated between 49.2°C and 57.9°C with a mean of 53.1°C and pH values varied between 7.9 and 8.5 with a mean of 8.3. The mean conductivity was 146.4 ± 8.3 µS/cm. The mean number of direct bacterial counts was $1.93x10^8$ bacteria / l. Overall, the bacterial cell counts were about 20% lower than in cold drinking water (Figure 3.1 b). Total bacterial counts in cold drinking water did not show a clear seasonal trend, i. e. no maxima or minima whith respect to seasons were observed (Figure 3.1 b). The dynamics of the hot water bacterial counts seemed to follow the cold water dynamics on a lower level until June 2009, with a lower variability with a mean around $1.5x10^8$ bacteria / l (drinking water).

We compared meteorological data, such as precipitation and temperature, with bacterial cell counts in the cold drinking water. The correlation analysis of the monthly mean of bacterial cell counts directly with the monthly precipitation resulted in a rather low Pearson correlation coefficient of -0.724 (data not shown). We decided to use another relation, because the absolute bacterial counts are a rather static parameter and the precipitation regime is more linked to the change of water parameters instead to static parameters. Precipitation mostly results in dilution or inflow of nutrients in the reservoir, not in the predefinition of nutrient concentrations. Thus, the monthly precipitation was put into relation to the monthly change of bacterial counts in cold drinking water (equivalent to the first derivative with respect to time, Δ(direct counts) / Δ(t)) (Figure 3.1 c).

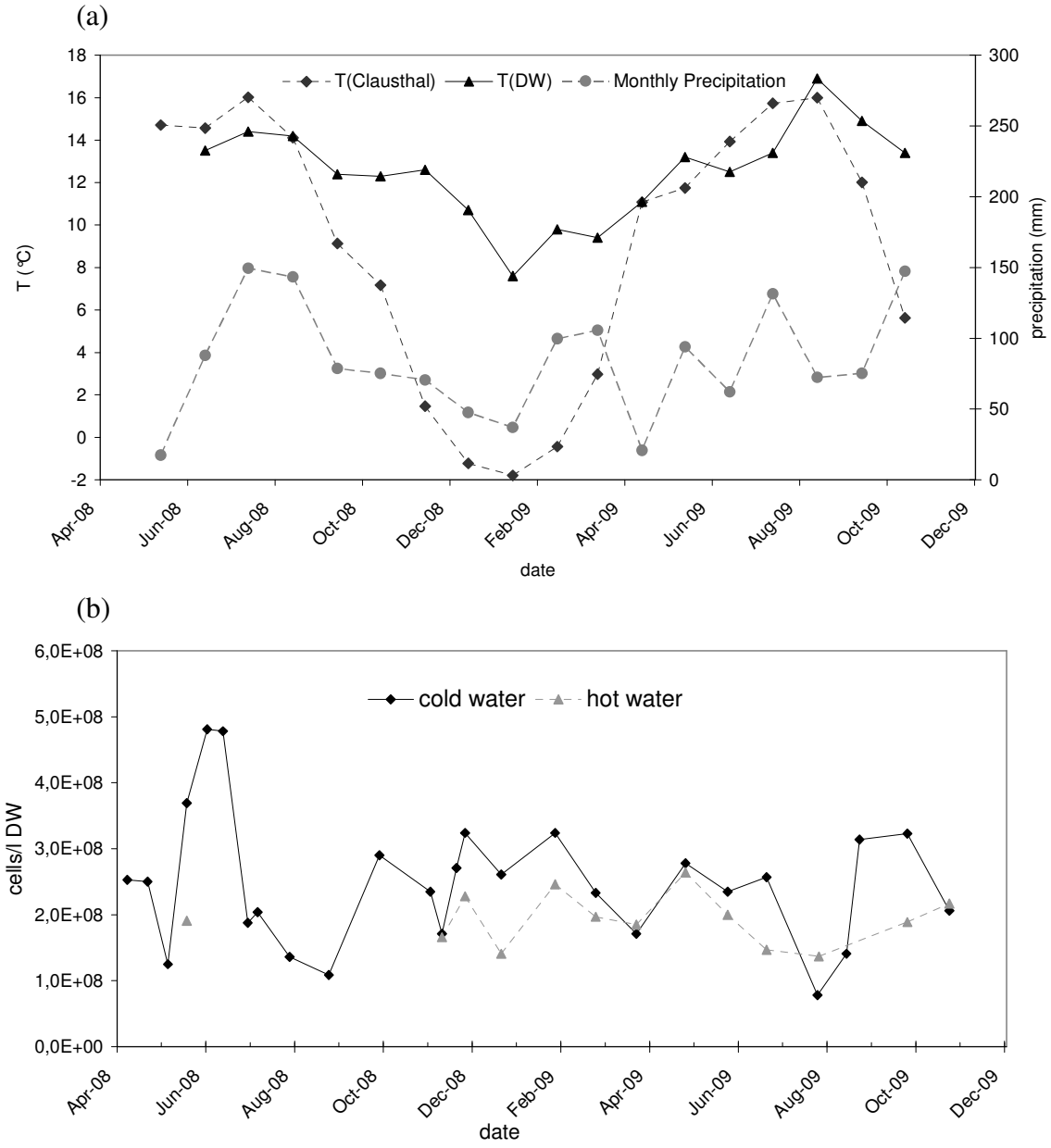
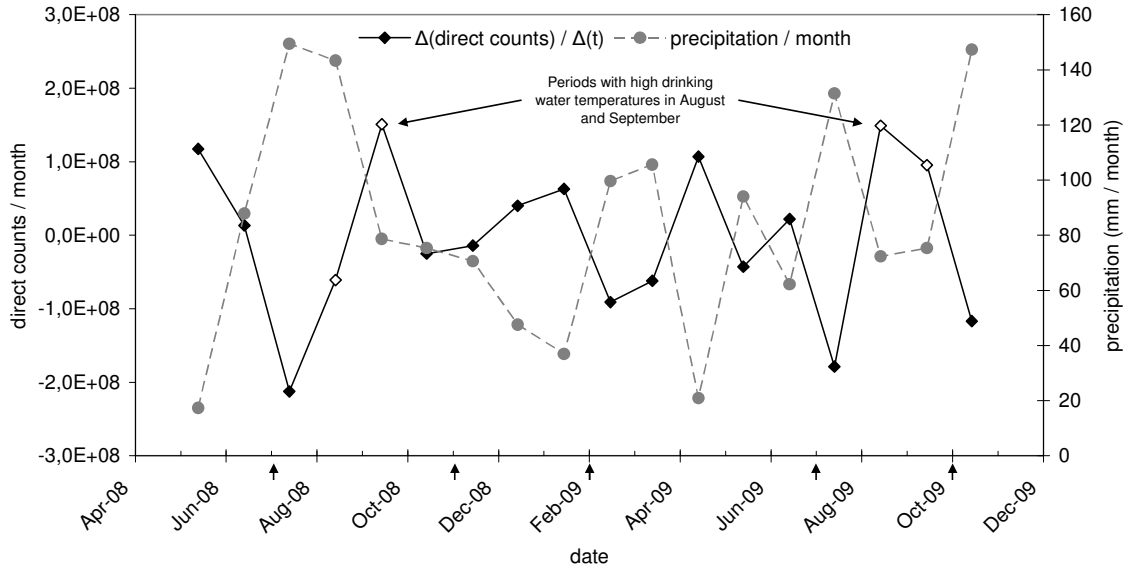
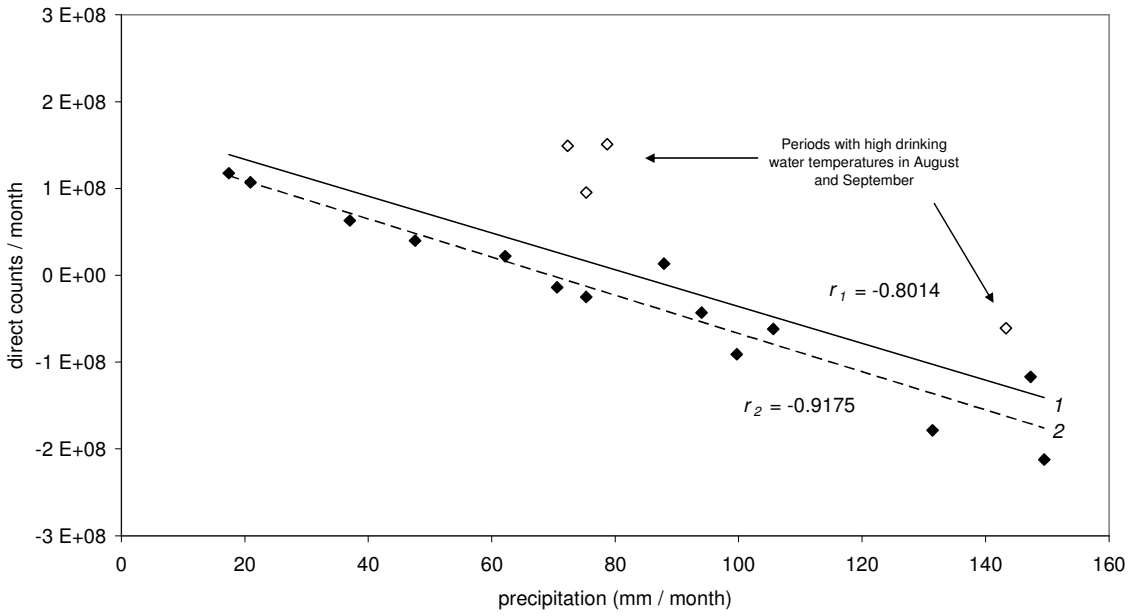


Figure 3.1: (a) Climate data of Clausthal-Zellerfeld representing climatic parameters for the cathment area of the Grane reservoir and the Ecker reservoir. (b) Total bacterial cell counts in cold and hot drinking water during 2008 and 2009. Continious line with diamonds: cold water; dashed line with triangles: hot water.

(c)



(d)



(c) Monthly precipitation and monthly change in total abundance of bacteria in cold drinking water. Arrows at the x-axis indicate a strong decrease in bacterial numbers and transition phases in the cluster analysis of corresponding RNA-based fingerprints (see Figure 3.2 d) (d) Correlation analysis (Pearson) between monthly precipitation and monthly change in total abundance of bacteria in cold drinking water. r_1: correlation coefficient including all outliers. r_2: correlation coefficient excluding four outliers belonging to months with high precipitation.

The curve of the monthly change in bacterial counts showed a clear inverse trend compared to the curve of the monthly precipitation in the catchment area of the reservoirs. In detail, a month of high precipitation is always accompanied by a strong decrease in bacterial numbers. A correlation analysis revealed a strong negative correlation between the precipitation and the change in bacterial counts in cold drinking water with a Pearson correlation coefficient of -0.801 (Figure 3.1 d, r_1). In this correlation curve, all four outliers to higher values of positive change in bacterial counts belonged to measurements in the month of August and September in both years. These months belonged to periods of a maximum in drinking water temperatures. Exclusion of these four outliers resulted in an even better correlation coefficient of -0.918 (Figure 3.1 d, r_2).

The monthly aerial mean temperature at Clausthal-Zellerfeld was an environmental parameter that influenced the temperature in cold drinking water as expected (Figure 3.1 b). The temperature of the cold drinking water followed the mean aerial temperature of the watershed region. In contrast, the temperature of hot drinking water did not show such a correlation and fluctuated irregularly due to technical measures such as heating and transfer. The number of bacteria measured in the hot drinking water, which is heated to 60°C, was only 20% lower than in cold, unheated drinking water. This was a rather unexpected result because the heating of drinking water to temperatures of 60°C is widely used to reduce total bacterial numbers and inactivate the number of pathogens like *Legionella* (43), A conceivable hypothesis for these unexpected high cell numbers could be: initially a decay of the bacterial microflora due to heating and then a regrowth of those bacteria that are not susceptible to high temperatures. Nutrient limitation is a major cause of limiting growth in drinking water (33). When cold drinking water is heated to hot drinking water, the killing of heat susceptible cells may lead to a release of organic and inorganic nutrients. The released nutrients may allow regrowth to comparable abundances bacteria had before. This could be the reason for similar dynamics of the hot water bacterial counts compared to the cold water bacterial counts until June 2009 despite their different community structure.

The strong negative correlation between the change in bacterial counts of cold drinking water and the precipitation regime could be seen as a dilution effect, i.e. with increased precipitation the water in the reservoir was diluted and the number of bacteria decreased what was presumably reflected in the reduced bacterial numbers of cold drinking water. This dilution effect seems to be in contrast to other studies, where

bacterial blooms in the reservoir were observed after heavy rain events (25). The increased amount of bacteria after heavy rain events was explained by run off causing an inflow of organic and inorganic nutrients into the reservoir system and a subsequent growth of bacteria due to a higher concentration of nutrients. In temperate regions runoff events are mostly short-term and accompanied by erosion of soil and sediments. In our study we did not correlate the change of bacterial numbers with the short term heavy rain events, but with the total volume of precipitation per month. In most cases, the precipitation was spread over the month without high precipitation events; thus, we assume an inflow of water with relatively low nutrient content into the reservoirs leading to a dilution effect. For our study, we assume that the precipitation regime is one of the most important environmental factors influencing the number of total bacteria in the drinking water.

Another important environmental factor influencing the concentration of bacteria was the drinking water temperature. Niquette et al reported a higher carbon uptake as a measure for bacterial activity and growth, when the water temperature exceeded approximately 15°C (35). The four major outliers to higher bacterial growth of the correlation analysis (Figure 3.1 d) occurred in month with the highest drinking water temperatures (around 15°C) in both summers 2008 and 2009. The regularity of these deviations is an indicator for the influencing property of temperature to the number of bacteria in drinking water.

Overall, we assume that not only the composition of the bacterial community of drinking water is influenced by the source water community, but also the amount of bacteria in drinking water by the bacterial concentration in the source water.

3.4.2 Dynamics of the community structure in cold drinking water

16S-rRNA and 16S-rRNA gene (rDNA) based SSCP fingerprints of samples taken in monthly intervals were used to analyse the community structure of cold drinking water. DNA-based fingerprints showed some constant bands and some with changing intensity over time, and only few suddenly appearing or vanishing bands (Figure 3.2 a). The comparative cluster analysis of the DNA-based fingerprints reflected different subclusters for different seasons (Figure 3.2 b), i. e. clear subclusters were found for spring, summer, and autumn/winter. Both summer subcluster of 2008 and 2009 showed closer similarities to each other than to those of other seasons. Additionally, the autumn/winter 2008/2009 subcluster and the spring 2009 subcluster showed similarities

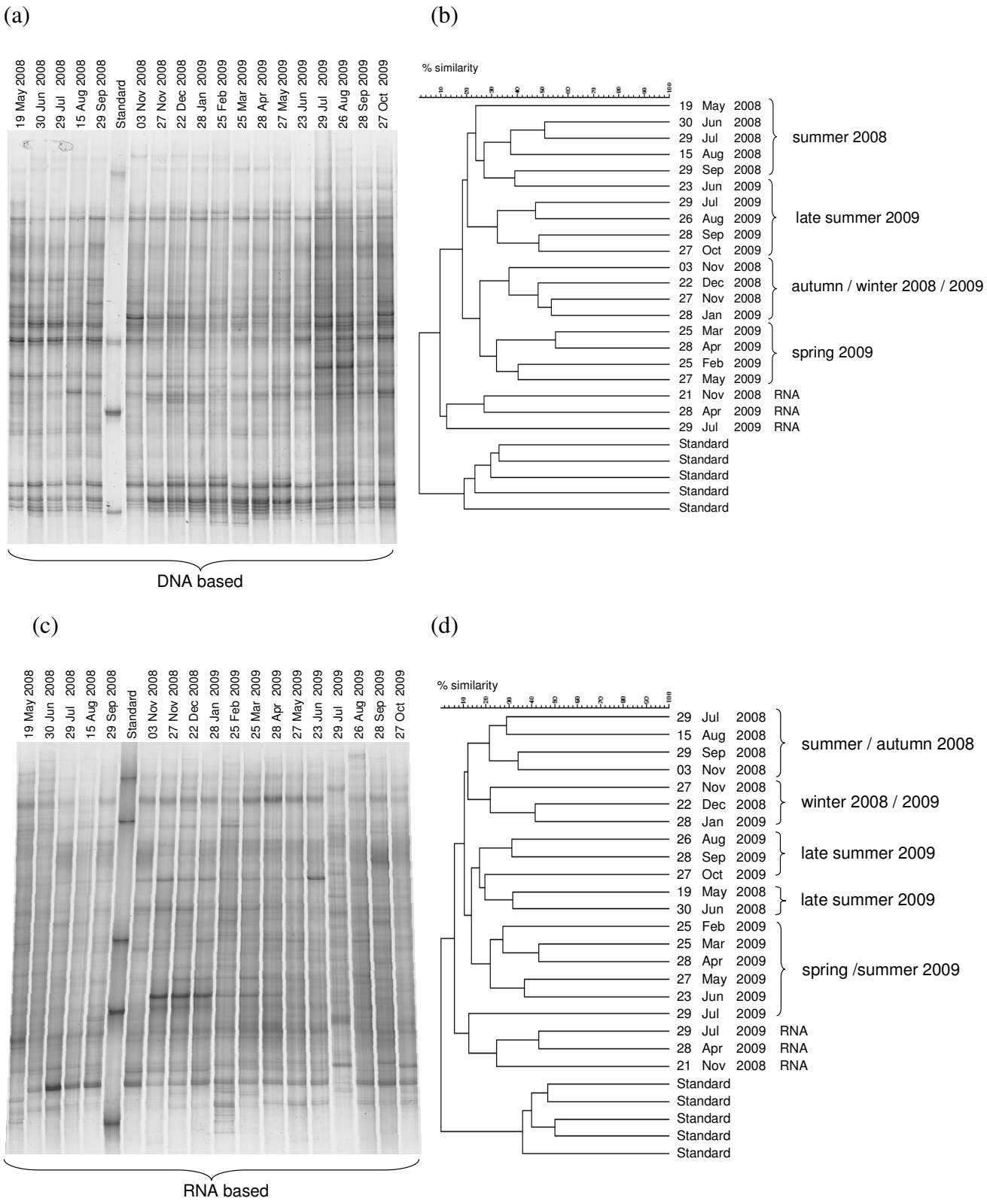


Figure 3.2: (a) 16S rRNA gene based SSCP fingerprints of cold water. (b) Comparative cluster analysis of DNA-based SSCP fingerprints of cold water. (c) 16S rRNA-based SSCP fingerprints of cold water. (d) Comparative cluster analysis of RNA-based SSCP fingerprints of cold water.

The main transition phases, in which the fingerprints switched from one seasonal subcluster into another, were in May/June and October/November.

RNA-based fingerprints showed few constant bands and many with changing intensity over time, and many suddenly appearing or vanishing bands (Figure 3.2 c). Again, different seasonal subclusters were found in the comparative cluster analysis of the RNA-based fingerprints (Figure 3.2 d). Subclusters of summer/autumn 2008, winter 2008/2009, late summer 2009, spring 2008, and spring/summer 2009 were found with transition phases between subclusters in June/July 2008, September/October 2008, January/February 2009, and June/July 2009 (indicated by arrows in Figure 3.2 d). Interestingly, these transition phases occurred simultaneously to local minima in the curve of the monthly change of direct counts and to months with high precipitation (over 100 l/m²) (Figure 3.1 c).

The RNA-based fingerprints showed highly different banding patterns compared to DNA-based fingerprints. Consequently, both datasets, RNA-based and DNA-based fingerprints, clustered separately as shown by a subset of three RNA-based fingerprints on the DNA-based gel and vice versa (Figure 3.2 b / d). The similarities between DNA-based and RNA-based fingerprints were below 10%. Additionally, RNA-based fingerprints showed a lower similarity to each other: in DNA-based fingerprints, the similarity between fingerprints differed between 18% and 56%, while in RNA-based fingerprints this range was only about 10% to 42%.

DNA- and RNA-based fingerprints of the cold drinking water were substantially different. Using the hypothesis that DNA-based bands represent the present bacteria and RNA-based fingerprints represented the active part of the community, we could conclude that those bacteria which had a high abundance were not necessarily those that were the active ones. Although the changes in the presence of bacteria in cold drinking water were moderate and slightly, changes in bacterial activity could be abrupt and intense. In both, DNA-based as well as in RNA-based fingerprints, summer clusters of 2008 and 2009 showed similarities to each other. These similarities revealed reoccurring similarities in seasonal drinking water community structures and community activities. Not only similarities of both types of fingerprints between different seasons were found, compared with former studies of the drinking water of the Harzwasserwerke the fingerprints bear a high resemblance to those of former studies (14, 17, 23). The running distances of several major bands from DNA and RNA-based fingerprints could be found even seven years ago in the drinking water originating from the same drinking

water supply. While some seasonal variation can be observed in short time periods, the resemblance to fingerprints of former studies emphasizes the stability of the cold drinking water community structure from year to year.

The cluster analysis of the DNA based fingerprints revealed transition phases between seasonal subclusters in spring and autumn. These phase transitions may refer to the mixing times of a normal dimictic reservoir. Due to the mixing of the reservoir, nutrients and bacteria are exchanged between the different layers which leading to a modified community structure (41), as reflected in our cold drinking water community. In contrast, transition phases of the bacterial community cluster of the RNA-based fingerprints did not coincide with those of the DNA based transition phases. Other environmental parameters seemed to affect the community structure revealed by RNA based. Time periods spanned by subclusters in the cluster analysis of the RNA-based fingerprints were consistent with time periods between months with high precipitation: e.g. between two periods of strong precipitation in February/March 2009 and in August 2009 we found a subcluster spanning time period of February until July 2009 (Figure 3.2 c, Figure 3.3 d, transition phases are indicated by small arrows in both figures). A month of high precipitation and thereby changed environmental parameters had presumably a strong impact on the activity of bacteria in cold drinking water and not only in the reservoir, irrespective of all water treatment for drinking water preparation, while the community structure itself was not affected by precipitation regime as revealed by DNA-based fingerprints (Figure 3.2 a).

3.4.3 Dynamics of the community structure in hot drinking water

For hot drinking water, the DNA-based fingerprints showed many constant bands and few with a changing intensity over time, and only very few suddenly appearing or vanishing bands (Figure 3.3 a). Separate subclusters reflecting different seasons were obtained by comparative cluster analysis of the DNA-based fingerprints (Figure 3.3 b). Three seasonal subclusters of winter/spring 2009, summer 2009, and autumn 2008 were found with similarities to each other varying between 25% and 52%. RNA-based fingerprints showed many constant bands and some with a changing intensity over time, and few suddenly appearing or vanishing bands (Figure 3.3 c).

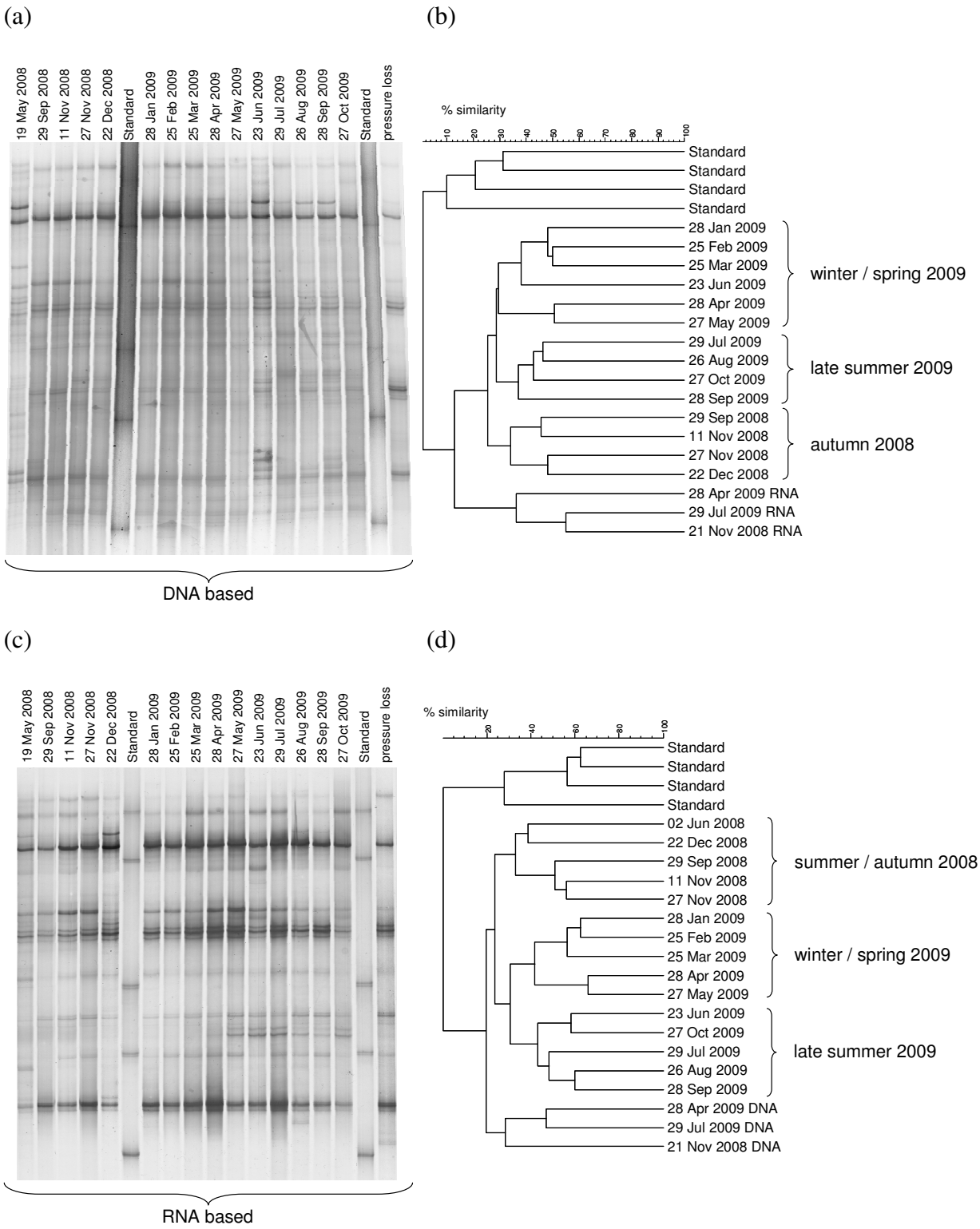


Figure 3.3: (a) 16S rRNA gene based SSCP fingerprints of hot water (b) Comparative cluster analysis of DNA-based SSCP fingerprints of hot water (c) 16S rRNA-based SSCP fingerprints of hot water (d) Comparative cluster analysis of RNA-based SSCP fingerprints of hot water.

Like in cold drinking water, three seasonal subclusters were found for summer/autumn 2008, winter/spring 2009 and summer 2009, with each containing five fingerprints (Figure 3.3 d). The similarities between RNA-based fingerprints varied between 22% and 62%. In hot water, RNA-based fingerprints showed very similar banding pattern to those of DNA-based fingerprints. In both types of fingerprints, five major constant bands at similar running distances were observed (Figure 3.3 a/c, indicated by arrows). These five bands only differed in intensity between RNA and DNA-based fingerprints. In general, RNA-based fingerprints showed higher intensities of these five bands, while other bands appeared in weaker intensities.

The seasonal variability of the present bacteria in hot drinking water was rather low, because the banding pattern in DNA based fingerprints did not vary much over time. In the RNA based fingerprints, the banding pattern showed a higher but still low variability. Nevertheless, for both kinds of fingerprints, fingerprints of the same season clustered together, indicating a weak temporal variation maybe influenced by parameters such as nutrient concentration or varying usage of hot water due to different seasons and therefore a prolonged residence time of the hot water in the tubes. For RNA based fingerprints, the five constant bands showed a higher intensity than in the DNA based fingerprints, indicating that few bacteria had a high activity in hot water. Similar banding patterns between DNA and RNA based fingerprints showed that the present bacteria, detected by DNA based fingerprints, were also active, as the same bands were found in the RNA based fingerprints, representing the active part of the community. This effect could be explained by regrowth: the cold water community was mostly destroyed by heating of the water up to 60°C, and subsequently only those bacteria adapted to high temperatures were growing to recolonize the hot drinking water, i.e. the thermophilic and thermotolerant bacteria.

3.4.4 Comparison of community structure of cold and hot water

The community structure of cold drinking water and hot drinking water differed substantially. Entirely different banding patterns for DNA based fingerprints and for RNA based fingerprints were obtained (Figure 3.2 a, c and 3.3 a, c). While in hot drinking water RNA and DNA based fingerprints were quite similar, they were totally different in cold drinking water. Differences of the community structures could also be found in rank abundance plots of the respective communities. We used relative band intensities from DNA based fingerprints as a measure for relative abundance. To obtain

comparable datasets we used a cutoff of 0.1% relative abundance, as this is the detection limit for reliable estimates of relative abundances. Regression analysis of this data resulted in different curves for cold and hot drinking water (Figure 3.4). For cold water, the course of the curve was rather flat and the detection limit was reached between the 46th and the 56th rank, with a mean rank of 52.4 (richness). The abundance of the most abundant phylotype varied between 7.4% and 16.2% (mean 10.0%). For hot drinking water instead, the curve was steeper and the detection limit was reached already between the 26th and 51st rank with a mean rank of 38.0 (richness). The abundance of the most abundant phylotype in hot drinking water reached levels between 20.9% and 38.3% (mean 28.6%).

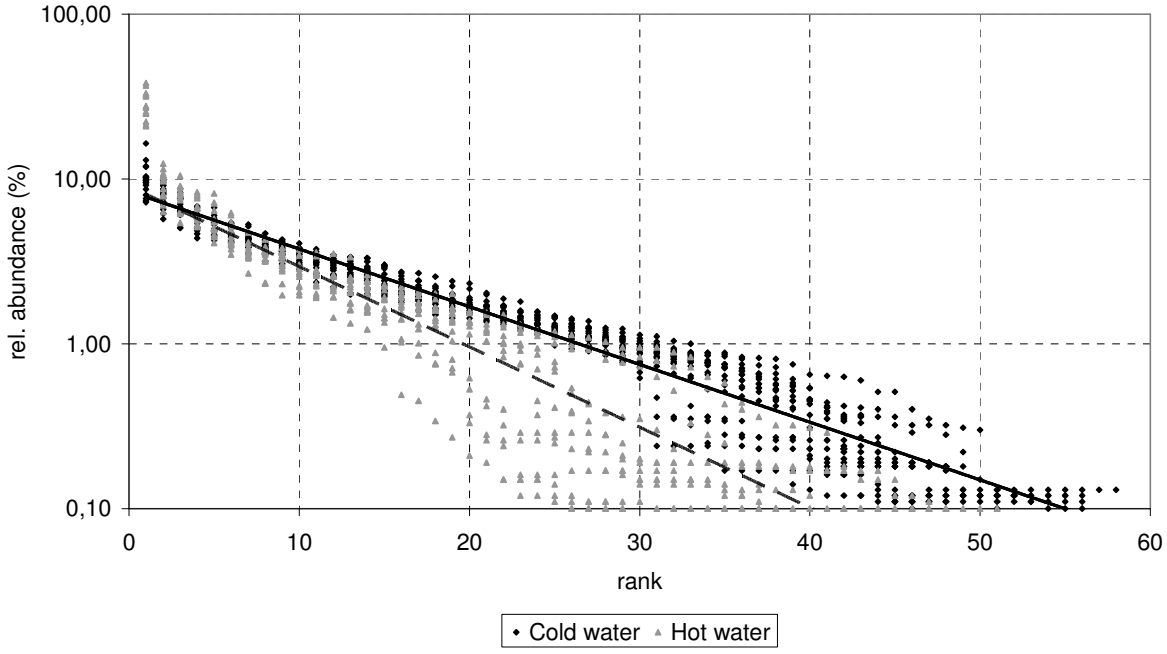


Figure 3.4: Rank abundance plot of cold water (black) and hot water (gray) using relative band intensities from DNA-based fingerprints as a measure for relative abundance. Lines show the regression analysis of plotted rank abundance curves. Continious line: cold water; dashed line: hot water.

It was shown before for domestic hot water that the ratio between HPC and AODC in hot water was approximately 20 times higher than the ratio in cold drinking water (4). Therefore, it became clear that there were substantial differences in the community structure of cold and hot drinking water. Consequently we observed clear differences in community measures between cold and hot drinking water. The mean richness of cold drinking water was markedly higher than the richness of hot water. In addition, in hot water the mean first rank abundance was clearly higher and the slope of the exponential regression in a logarithmic plot was clearly steeper than these measures in cold drinking water.

As the hot drinking water forms an environment with extreme parameters (8), only few species may be adapted to it causing a low richness. These adapted species may grow in this environment without grazing and competition leading to high abundances. In contrast, cold drinking water provides an environment with common parameters. Many species are adapted to those parameters and therefore a complex community structure may have formed, indicated by a high richness. Possibly, as many species compete in cold drinking water for the same resources the relative abundances were lower than those in hot drinking water. Generally these differences in diversity measures were strong indicators for a markedly different bacterial community structure in cold and hot drinking water.

3.4.5 Taxonomic composition of the cold drinking water community

Excising and sequencing of the major SSCP-bands (approximately all bands above 0.1% relative band intensity) of cold drinking water fingerprints and subsequent alignment of the obtained sequences resulted in a set of 43 unique phylotypes. 23 phylotypes were obtained from DNA-based fingerprints, and 27 phylotypes from RNA-based fingerprints, with 7 identical phylotypes among DNA-based and RNA-based sequences. With the about 400nt long sequences we could resolve up to the species level. This taxonomic resolution should be sufficient for the distinction of environmental phylotypes that often have no closely related cultured neighbour species.

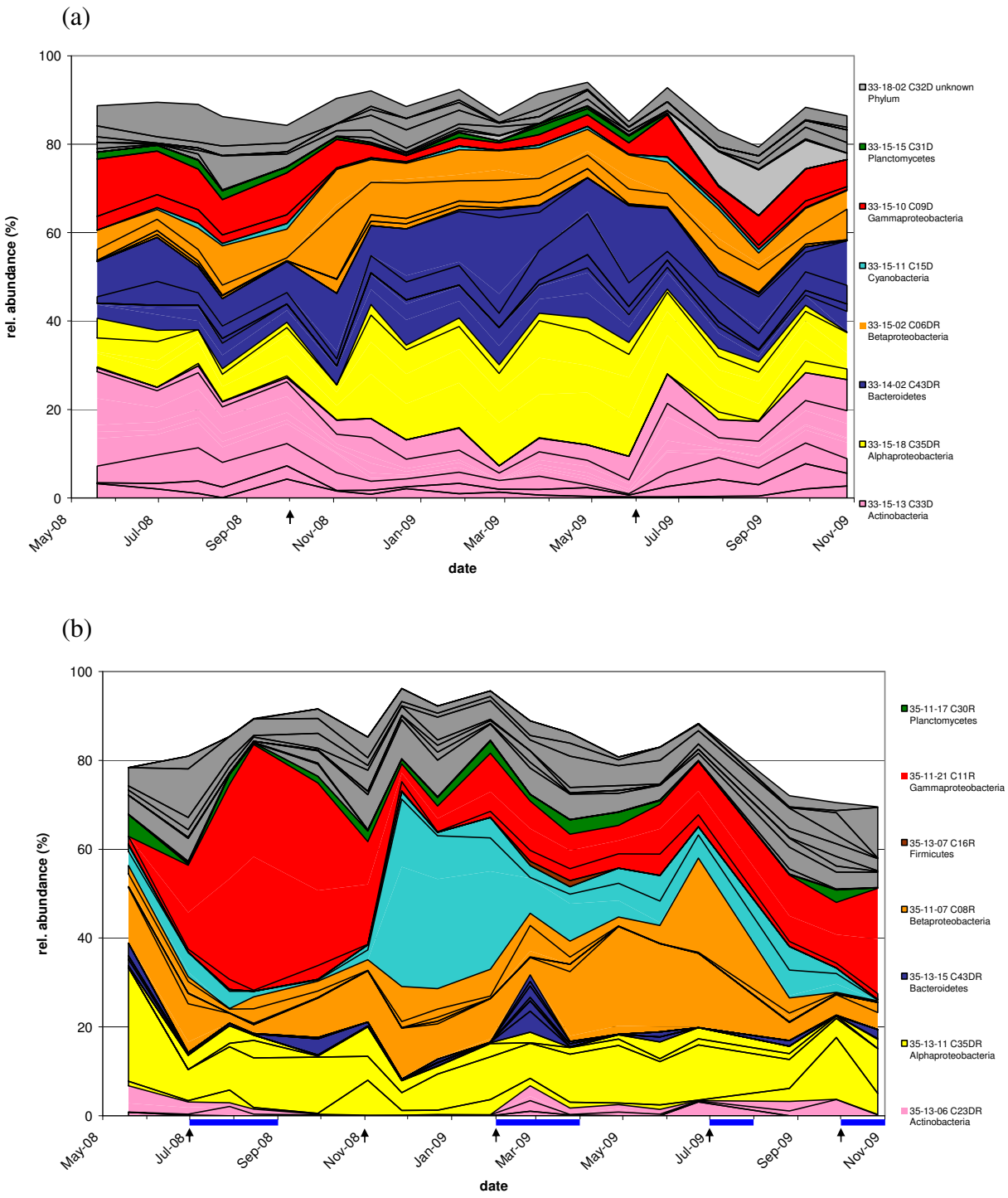


Figure 3.5: Seasonal variation of relative abundances of the phylotypes obtained from cold water. The colors are corresponding to the major phylogenetic groups. Phylotypes are separated by solid lines. Arrows indicate phase transitions of the cluster analysis of the respective fingerprints. Blue bars at the x-axis indicate months with high precipitation. (a) Phylotypes from the DNA-based SSCP fingerprints. (b) Phylotypes from the RNA-based SSCP fingerprints.

Table 3.1: Number of retrieved phylotypes from cold water and hot water (presence/absence data). DNA: Number of phylotypes found in DNA-based fingerprints. RNA: Number of phylotypes found in RNA-based fingerprints. DNA+RNA: Number of phylotypes found in both types of fingerprints.

Phylum / Class	**Cold water**				**Hot water**			
	DNA	**RNA**	**DNA+RNA**	**Total**	**DNA**	**RNA**	**DNA+RNA**	**Total**
Alphaproteobacteria	3	5	1	7	2	1	1	2
Betaproteobacteria	4	4	1	7	3	5	2	6
Gammaproteobacteria	2	3	0	5	0	2	0	2
Acidobacteria					1	3	1	3
Actinobacteria	5	5	1	9	1	0	0	1
Bacteroidetes	6	6	4	8	5	1	1	5
Cyanobacteria	1	2	0	3				
Firmicutes	0	1	0	1				
Planctomycetes	1	1	0	2	1	1	1	1
unknown	1	0	0	1				
total	23	27	7	43	13	13	6	20

The phylogenetic identification of obtained phylotypes is summarized in Table 3.1. The most common phylotypes belonged to the *Actinobacteria* phylum (9 phylotypes, 21%), found in both, DNA based and RNA based fingerprints, with only one phylotype in both types of fingerprints. Also *Bacteroidetes* were quite common (8 phylotypes, 19%) with four phylotypes found in both, DNA and RNA based fingerprints. Only *Alphaproteobacteria* (7 phylotypes, 16%) and *Betaprotebacteria* (7 phylotypes, 16%) also had at least one phylotype in common between DNA based and RNA based fingerprints. Other observed phylotypes were *Gammaproteobacteria* (5 phylotypes, 12%), *Cyanobacteria* (3 phylotypes (3 phylotypes, 7%), *Planctomycetes* (2 phylotypes, 5%) and *Firmicutes* (1 phylotype, 2%).

Relative abundances of the single phylotypes of a mean abundance above 0.1% detected in DNA-based fingerprints were estimated by band intensities and plotted against time (Figure 3.5 a). Two main phases of the bacterial community composition could be distinguished, winter and summer, whose beginning and ending coincide with the transition phases between the seasonal subclusters (Figure 3.2 b, Figure 5 a). The winter phase began in October 2008 and ended in May 2009. It is characterised by an

increased abundance of *Alphaproteobacteria* (25.4%) and *Bacteroidetes* (27.2%), but decreased abundance of *Actinobacteria* (12.7%) and *Gammaproteobacteria* (2.2%). The summer phase ended in September 2008 and began in June 2009. Its phylogenetic composition is characterized by an increased abundance of *Actinobacteria* (24.5%) and *Gammaproteobacteria* (9.6%), but decreased abundance of *Alphaproteobacteria* (12.3%) and *Bacteroidetes* (16.7%). During the whole sampling period, the abundances of *Betaproteobacteria* (12.0%), *Cyanobacteria* (0.6%) and *Planctomycetes* (1.0%) stayed rather constant.

The 16S rRNA abundance plot of the RNA-based fingerprints showed an entirely different picture (Figure 3.5 b). At least three clearly separated main phases could be identified with elevated rRNA abundances of only a single phylotype. The beginning and ending of each phase also coincided with the transition phases between the seasonal subclusters (Figure 3.2 d, Figure 3.5 b). The first phase began in July and ended in October 2008. Its beginning coincided with two months of high precipitation in July / August 2008 and a pronounced decrease in the change of total bacterial numbers in July 2008. It was characterized by an increased rRNA abundance of *Gammaproteobacteria* (42.3%), but decreased rRNA abundance of *Actinobacteria* (1.2%), *Cyanobacteria* (2.3%) and *Betaproteobacteria* (9.6%). The phase of high *Gammaproteobacteria* rRNA abundances comprised only one phylotype, C11R, which was only found in RNA-based fingerprints. The second phase began in November 2008 and ended in February 2008. Its beginning coincided with a strong decrease in the change of total bacterial numbers in October 2008 and a phase transition in the bacterial community reflected by DNA-based fingerprints. It was characterized by an increased rRNA abundance of *Cyanobacteria* (42.3%), and decreased rRNA abundance of *Actinobacteria* (1.8%), *Gammaprotobacteria* (10,0%) and *Betaproteobacteria* (16.7%). The phase of high *Cyanobacteria* rRNA abundances comprised only one phylotype, C13R. The third phase began in March 2009 and ended in June 2009. Its beginning coincided with months of high precipitation in February / March 2009 and a pronounced decrease in the change of total bacterial numbers. It was characterized by an increased rRNA abundance of *Betaproteobacteria* (27.8%) and *Actinobacteria* (2.3%), but decreased rRNA abundance of *Cyanobacteria* (10.4%) and *Gammaproteobacteria* (12.7%). The phase of high *Betaprotebacteria* rRNA abundances comprised two phylotypes, C01R and C08R, both were only observered in RNA-based fingerprints. The ending of the third phase again coincided with a month of high precipitation in July 2009 and a strong

decrease in the change of direct bacterial counts in July 2009. During the whole sampling period, i.e. for all phases, the rRNA abundances of *Planctomycetes* (1.7%), *Bacteroidetes* (1.5%) and *Alphaproteobacteria* (15.8 %) remained approximately constant.

Based on the hypothesis, that DNA based fingerprints represent the present bacteria, two main phases of the present bacterial community were observed. Transitions from one seasonal community to the other coincided with times of mixing events in dimictic lakes in spring and autumn. It can be inferred that the bacterial community changes in cold drinking water reflected the community changes in the dimictic reservoirs. The changes of environmental conditions like temperature, nutrients and carbon availability through mixing induce a remarkeble shift in the bacterial community of the reservoir which are still detectable in the drinking water community despite water treatment. Another well known effect occuring in freshwater ecosystems in the summer was also observerved in cold drinking water: the amount of *Actinobacteria* doubled in the summer phase compared to the winter phase (15, 16, 22). This effect is explained by the small, grazing resistant size of *Actinobacteria*, which allows for better carbon competition during high primary production phases.

Assuming that the rRNA-level is associated with the activity of the respective phylotype, phases of activity of single phylotypes are temporally limited and clearly separated. The coincidence of pronounced decreases in the change of total bacterial numbers with every phase transition and the coincidence of months of high precipitation with almost every phase transition could hint of a causal relationship. Possibly the dilution effect of the water in months with high precipitation or other environmental impacts like the mixing of the reservoir created different environmental conditions leading to a collapse of the existing bacterial community, which was detected by a lower number of bacteria. Subsequently, the community could regrow to higher levels until the next month of heavy rainfall. Interestingly, these phases of high activity were only observed in single phylotypes and not in all members of the taxonomic group. This could mean that these phylotypes were highly specialized to the respective environmental conditions.

An unexpected result was the high relative amount of 16S rRNA of the phylotype C13R in the RNA-based fingerprints during the winter. As this phylotype belongs to the photoautotrophic *Cyanobacteria*, order *Synechococcus*, it was thought to have its highest activity in the summer, where most cyanobacterial blooms are normally

observed. High amounts of 16S rRNA in *Cyanobacteria*, especially for *Synechococcus*, were observered before. This high level of rRNA might be explained by a storage function of RNA for phosphorus and nitrogen in *Cyanobacteria* (3, 13, 26).

Comparing abundances of the DNA-based fingerprints with those of the RNA-based fingerprints, it can be inferred that most active bacterial phylotypes were those that are not detectable in DNA based fingerprints. Only 7 out of 43 phylotypes (16%) were found in both types of fingerprints. In addition, some taxonomic groups tended to possess low rRNA-levels despite their high occurrence on DNA-based fingerprints (e.g. *Actinobacteria* and *Bacteroidetes*). Especially for *Actinobacteria* in drinking water reservoirs the low content of ribosomes was already described as a result of their small size, resulting in a lower detection rate also by other molecular detection methods like FISH (15, 34).

Despite their high abundance in RNA based fingerprints, highly active phylotypes only had low numbers of rRNA copies. According to the rrnDB Database (29), the number of rRNA operons in the taxonomic groups of the nearest cultured neighbour of the high active phylotypes did not exceed three. This could be a hint to explain the low amount of rRNA genes detected in the DNA-based fingerprints.

3.4.6 Taxonomic composition of the hot drinking water community

We detected a total of 20 bacterial phylotypes in the hot drinking water community. 13 of these phylotypes were found in DNA-based analyses, 12 phylotypes in RNA analyses and 6 phylotypes in both, DNA and RNA-based analyses (Table 3.1). Although the comparative cluster analysis of the DNA-based hot drinking water fingerprints assigned a community pattern according to seasons, no definite seasonal phases were observed in the dynamics of the abundances of phylotypes. The hot water community was characterized by five dominating taxonomic groups. In detail, we found *Betaproteobacteria* (32.4%), *Acidobacteria* (14.7%), *Alphaproteobacteria* (13.7%), *Bacteroidetes* (6.7%) and *Planctomycetes* (5.4%) forming the core community (72.9%). Each group was dominated by a single phylotype which did not show significant seasonal variations in its abundance (Table 3.2).

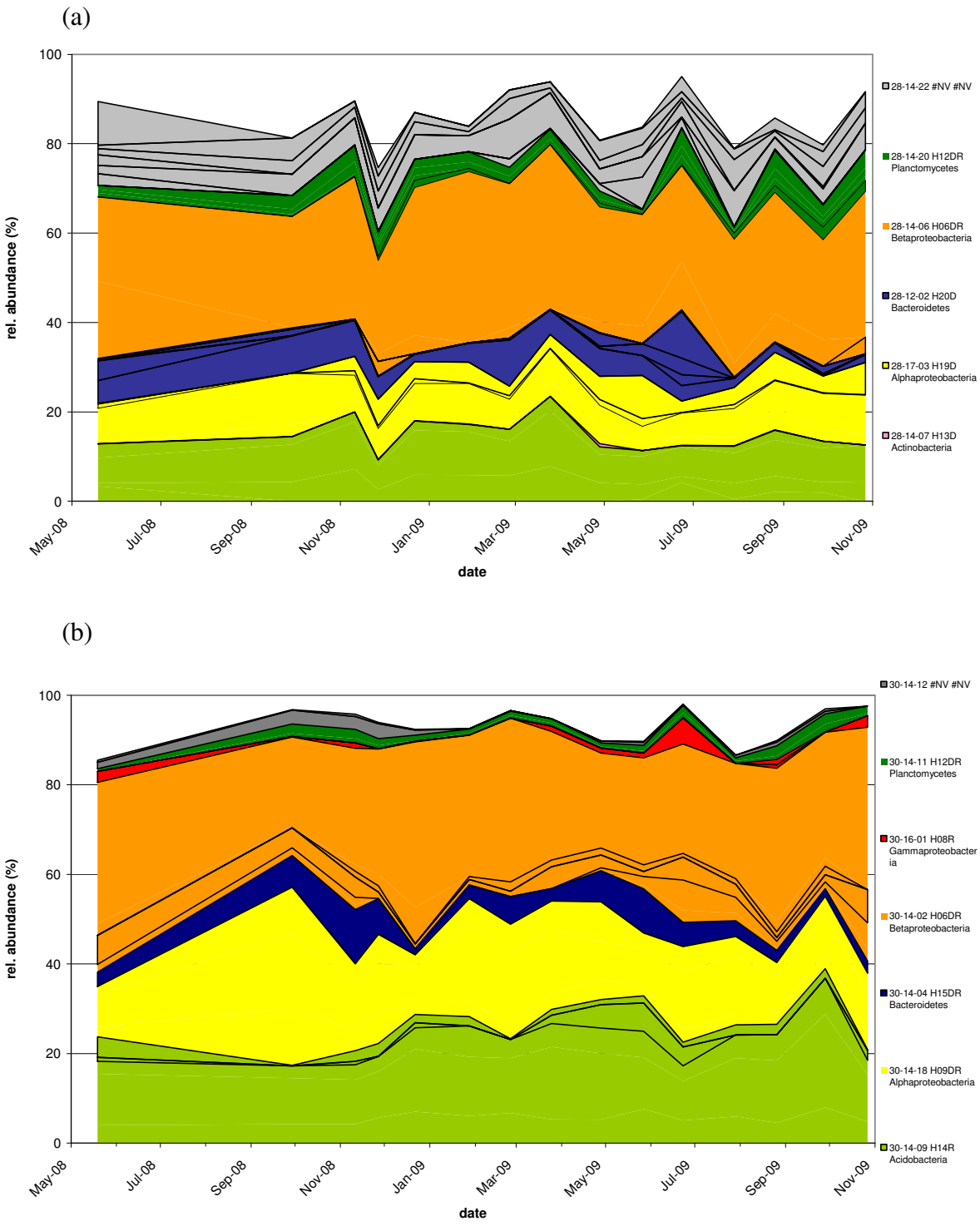


Figure 3.6: Seasonal variation of relative abundances of the phylotypes obtained from hot water. The colors are corresponding to the major phylogenetic groups. Phylotypes are separated by solid lines. (a) Phylotypes from the DNA-based SSCP fingerprints. (b) Phylotypes from the RNA-based SSCP fingerprints.

Similar to the DNA-based fingerprints two main phases were detected in the cluster analysis of the RNA-based fingerprints, but no clear seasonal variation in the abundances of phylotypes was observed (Figure 3.6 b). The abundance of all taxonomic groups like *Betaproteobacteria* (36.7%), *Acidobacteria* (26.2%), *Alphaproteobacteria* (20.6%), *Bacteroidetes* (5.1%), *Planctomycetes* (2.0%), and *Gammaproteobacteria* (1.2%) stayed rather constant. Also on the RNA-based fingerprints, the same five dominating phylotypes were building the core community.

Considering the presence/absence data of single phylotypes, 6 phylotypes (30%) were found on both, DNA and RNA-based fingerprints. Most taxonomic groups had comparable abundances on both types of fingerprints, e.g. the *Betaproteobacteria:* 32.4% (DNA) and 36.8% (RNA), or *Bacteroidetes:* 6.7% (DNA) and 5.1% (RNA). For both, DNA and RNA-based fingerprints, five main phylotypes belonging to five different taxonomic groups were observed characterizing the hot water community. Phylotype H06DR (*Betaproteobacteria*), H09DR (*Alphaproteobacteria*), H10DR (*Acidobacteria*), H15DR (*Bacteroidetes*) and H12DR (*Planctomycetes*) represented the main bands of both types of fingerprints (DNA total abundance: 65.4%, RNA total abundance: 80.9%). The only discrepancy between DNA and RNA-based fingerprints on the phylum level were the *Gammaproteobacteria* (1.2%), which were only present on the RNA-based fingerprints.

Assuming a similarity of 90% or higher for 16S rRNA gene similarity with a species found for example in hot springs, volcano mud or hydrothermal deposits, the phylotype was rated as “of hot habitat origin”. In total, 45% of all phylotypes in hot drinking water were of “hot habitat origin”, whereas 20% of all phylotypes found in hot drinking water had also been observed in the cold drinking water of the DWSS Harzwasserwerke in former studies (14, 23).

In hot drinking water no or only very low seasonal effects on the bacterial community composition were observed in both types of fingerprints. An explanation for this could be the constant temperature. Due to the insulation of the pipes and the fact that major parts of the hot water distribution system is located inside of buildings, environmental factors like temperature could influence the hot water only very little. The distribution of taxonomic groups is rather similar between DNA- and RNA-based fingerprints. Thus, using again the hypothesis that the rRNA abundance reflects the activity, it can be inferred that the present community in hot drinking water was also active. Possibly, those bacteria being able to survive the hot water conditions needed

permanent activity to keep their cell processes running. Interestingly, this constant community consisted of only five phylotypes belonging to five taxonomic groups, but these had very high abundances and activity, showing that only few bacteria were adapted to these hot temperatures. Two effects may influence the building of such a community: 1) Selection of thermophilic bacteria from the seedbank and 2) selection of thermotolerant phylotypes from the core community of the cold drinking water.

Most of the phylotypes were assigned to be of “hot habitat origin”, although the source of the hot drinking water did not have any contact to hot habitats. These phylotypes were considered to be thermophilic. This distance to any hot habitats supports the model of "abundant and rare members" describing the bacterial community composition in pelagic environments. In this model, the community consists of a core community with few taxa that are highly abundant and a huge seed bank with nearly infinite numbers of very low abundant phylotypes (18, 21, 37). As the hot drinking water provides niches that differ from those in the cold drinking water, we hypothesize that the low abundant thermophilic bacteria from the cold drinking water seed bank were recruited forming partly the core community in hot drinking water.

The second most prevalent group consists of phylotypes already found in the cold drinking water of the Harzwasserwerke. Thus, a selection for those thermotolerant bacteria of the cold drinking water community happened, that were able to survive water temperatures of 60°C. This shows that the reservoir community influences via the cold drinking water community even the hot drinking water community.

Table. 3.2: Composition of taxonomic groups and their dominating phylotypes in hot drinking water revealed by DNA and RNA-based fingerprints. Phylotypes marked with D only occured in DNA-based fingerprints, marked with R only in RNA-based fingerprints, and phylotypes marked with DR occured in both.

DNA				RNA			
Phylum / class	**Rel. abundance**	**Phylotype**	**Rel. abundance**	**Phylum / class**	**Rel. abundance**	**Phylotype**	**Rel. abundance**
Acidobacteria	14.74	H10DR	14.7	*Acidobacteria*	26.3	H10DR	23.0
						H11R	1.5
						H14R	1.7
Actinobacteria	0.09	H13D	0.1				
Alphaproteobacteria	13.67	H09DR	9.0	*Alphaproteobacteria*	20.6	H09DR	20.6
		H19D	4.7				
Bacteroidetes	6.70	H15DR	4.6	*Bacteroidetes*	5.1	H15DR	5.1
		H16D	0.5				
		H17D	0.5				
		H18D	0.9				
		H20D	0.2				
Betaproteobacteria	32.37	H06DR	31.8	*Betaproteobacteria*	36.8	H06DR	30.2
		H03DR	0.6			H03DR	3.1
		H01D	0.02				
						H02R	2.4
						H04R	1.0
				Gammaproteobacteria	1.2	H08R	1.2
Planctomycetes	5.39	H12DR	5.4	*Planctomycetes*	2.0	H12DR	2.0

3.4.7 Comparison of community composition in cold and hot drinking water

Clear seasonal patterns with correlation to environmental parameters like precipitation and temperature in both, DNA and RNA-based fingerprints, were only found for the cold drinking water community. In the hot water community only weak or no seasonal variation in the community structure was detected. In cold drinking water community, one phylotype was found in DNA-based fingerprints dominating all others in the winter period (C34D, *Alphaproteobacteria*), while in RNA-based fingerprints at least three single phylotypes were dominant over phases for several months (C11R, *Gammaproteobacteria*; C13R, *Cyanobacteria*; C01R, *Betaproteobacteria*). All dominant RNA phylotypes could not be found in DNA-based fingerprints and vice versa.

In hot drinking water, the community was less rich, i.e. it was dominated by few phylotypes with higher abundances. On both, DNA and RNA-based fingerprints, the same five phylotypes were found to dominate the phylum, and therefore the whole community. These five phylotypes H06DR (*Betaproteobacteria*), H09DR (*Alphaproteobacteria*), H10DR (*Acidobacteria*), H15DR (*Bacteroidetes*) and H12DR (*Planctomycetes*) did not show a seasonal variation and were different to those temporarily dominating the cold drinking water community.

Actinobacteria, which were very common in DNA-based fingerprints, and *Cyanobacteria*, temporarily very common in RNA-based fingerprints, only occurred in cold drinking water community. In hot water communities, the phylum of *Acidobacteria*, which was very common in both, DNA and RNA-based fingerprints, was unique.

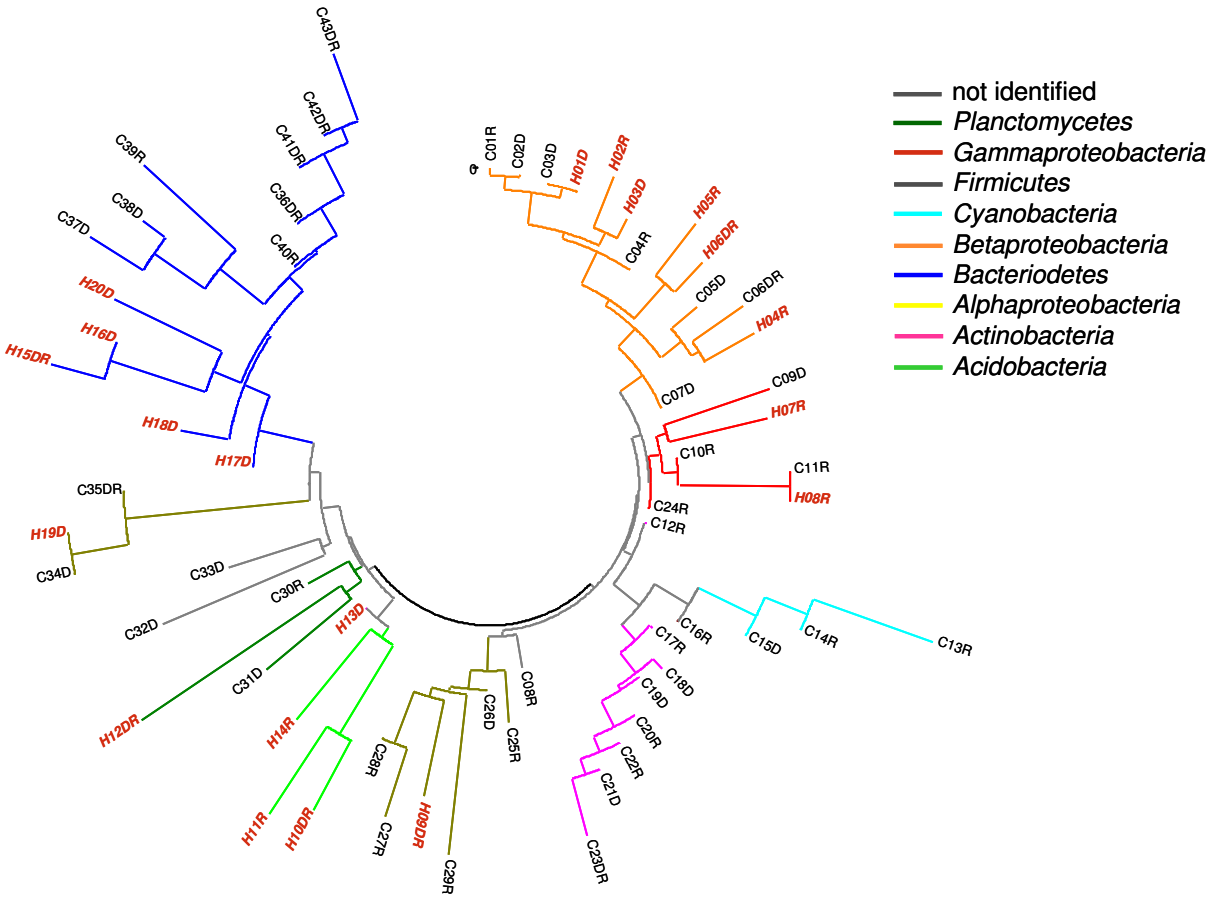


Figure 3.7: Phylogenetic tree of all cold water and hot water phylotypes. Line colour indicates the respective phylum. Cxx: Cold water water phylotype; Hxx: Hot water phylotype (red letters); D originated from DNA-based fingerprint; R: originated from RNA-based fingerprint. The taxonomic tree was inferred using the Neighbor-Joining method. Evolutionary distances were computed using the Maximum Composite Likelihood method. Phylogenetic analyses were conducted in MEGA4.

The community composition of cold and hot drinking water differed substantially. Only three common phylotypes between cold and hot drinking water could be observed (Figure 3.7): C03D / H01D (*Betaproteobacteria*), C11R / H08R (*Gammaproteobacteria*), and C34D / H19D (*Planctomycetes*). All these phylotypes showed low abundances (0.1% - 4%) in hot water fingerprints.

In hot drinking water the core community in DNA based and RNA based fingerprints was very similar. Therefore we conclude that the relative abundance of a phylotype in hot drinking water also reflects that it is active. Presumably, in hot drinking water a high amount of 16S rRNA is an indicator for activity and growth. However, in cold drinking

water the situation was different: the communities and the dynamics of the communities were substantially different in DNA and RNA based fingerprints. The high abundant bacteria were not necessarily the actives ones, while the active bacteria were not necessarily of high abundance. We assume that the activity of cold drinking water bacteria is dependent on different factors than the activity of hot water bacteria. While the dynamics of the rRNA abundance in cold drinking water is strongly influenced by environmental parameters such as precipitation and temperature, the dynamics of the hot drinking water are not or only on a very low level influenced by these parameters. Although it can be assumed that the organic and inorganic nutrient levels in hot water had not considerably changed during the heating process, the dynamics of the activity of the hot drinking water community seem to be less dependent on changes in environmental conditions than the dynamics of the activity of the cold drinking water community.

Some phylogenetic groups such as *Actinobacteria* in cold water and *Acidobacteria* in hot water seemed to be typical for the respective community as they were not found in the other community. Two of the three phylotypes that were found in both, cold and hot drinking water (C34D and C11R) belonged to the most abundant phylotypes observed in the cold drinking water either on the DNA based or on the RNA based fingerprints respectively. Therefore, they presumably originated from damaged cells or nucleic acids still present in the hot drinking water after heating procedure, but still detectable by the SSCP.

3.5 Conclusions

This is the first study analysing the seasonal dynamics of the community composition and its activity of cold and the corresponding hot drinking water in relation meteorological data of the catchment area. It was demonstrated that the precipitation in the catchment area was the major impact influencing the amount of bacteria in cold drinking water. Another factor influencing the amount of bacteria was the temperature of the cold drinking water. The community structure of the present bacteria in cold drinking water may have been changed by mixing events in dimictic reservoirs, but it turned out that highly abundant bacteria were not necessarily the active ones and vice versa. The activity of the cold drinking water community was also mainly influenced by

months of high precipitation, characterized by phases of high activity of only a single phylotype.

For the hot drinking water, which was prepared from the cold drinking water by heating to 60°C, the amount of bacteria in the hot drinking water was only 20% lower than that of cold drinking water bacteria, possibly due to regrowth. The hot drinking water community was less rich than cold drinking water community and was mainly composed of thermophilic and thermotolerant bacteria that were also found in hot environments. In contrast to cold drinking water, the hot drinking water community composition was not influenced by environmental factors like precipitation or temperature. In hot drinking water, the relations of abundances of phylotypes were also reflected by their activity, i.e. highly abundant bacteria were also highly active.

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Chapter 4

4 Comparison of overall structure and composition of bacterial communities in mature drinking water biofilms and bulk water of a local network

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4.1 Abstract

Central to the understanding of health risks stemming from drinking water is the relationship between bacteria in bulk water and biofilm attached to the pipes of the supply system. To this end, a detailed analysis of the whole bacterial community in bulk water and corresponding biofilms was done concentrating on an over 20 year old drinking water supply system (DWSS). This DWSS was studied using cultivation-independent 16S rRNA fingerprints based on extracted DNA and RNA from bulk water and biofilm samples. The overall community structure of the bacteria in the bulk water was the same across the city of Braunschweig whereas the bacteria in all biofilm samples showed a highly different structure of each sample. This was also reflected in the community composition which was very similar for all bulk water samples whereas all biofilm samples contained a unique community with no overlap to the phylotypes observed in bulk water. Only biofilm communities sampled on nearby sampling points showed similar communities in spite of different support materials. In the bulk water the active (RNA-based) bacterial fraction was substantially different from the present (DNA-based) bacterial fraction, whereas in biofilms both were more similar to each other, indicating that most species present in drinking water biofilms were also active. In addition, all biofilm communities showed higher relative abundances of single phylotypes with a reduced richness, i.e. the total number of all phylotypes above the detection limit, compared to bulk water communities. Overall, we hypothesize that during several years, physically related biofilm communities will show similar community structures. This behaviour suggests, with respect to the diversity and structure of biofilm communities that, there are similar mechanisms structuring these communities in DWSS, i.e. all biofilms provide a similar number of niches but are filled with different species. (293w, 250w max)

4.2 Introduction

Biofilms are present in every drinking water supply system (DWSS), commonly attached to the surface of tubing material of the distribution network (3). Biofilms can be of great relevance for public health, because many potentially pathogenic bacteria are not located in the bulk water but in the biofilm of the pipes, where they are more protected against adverse environmental conditions such as disinfection measures (7, 39, 45). Especially in the case of pressure loss events, the shear stress can disrupt pieces

of biofilm causing not only an unpleasant colour and flavour of the bulk water but also a potential health risk (10, 38).

Many studies have focused on the examination of artificial drinking water biofilms in model systems (23, 27, 30). Assuming that only minor changes occur after a rather stable biofilm developed, most biofilm studies concentrated on short-term studies with biofilms grown for only a few months. However, Martiny et al. (30) showed that a stable community in a drinking water biofilm needed years to be established. They describe in their model DWSS a four phase succession of a drinking water biofilm: In the first 14 days, a biofilm is formed by bacteria recruited from the planktonic population in bulk water. In the second phase, during the first eight month, cell numbers increase and the biofilm community is dominated by members of the phylum *Nitrospira*. The third phase is dominated by a change to a distinct community and the disappearance of the dominating *Nitrospira* during two years. In their model DWSS, the last phase is reached after three years and the mature biofilm consisted of a mix of heterotrophic and autotrophic bacteria with a rather even community structure. This four phase model was developed for biofilm growing on stainless steel. However, for young biofilms it is reported that different tubing material of model DWSS, such as copper, PVC or stainless steel, may affect the number of cells, the morphology, and the bacterial composition (10, 25, 39, 40). It has been reported for various pathogens, such as *Legionella pneumophila*, *Mycobacterium* spp., and *Helicobacter* spp. that they were primarily associated with or grow in biofilms (4, 10, 12, 33). Therefore, drinking water biofilms can function as an important reservoir for pathogens and may provide a source of bulk water contamination by exchange of bacteria between biofilm and bulk water (26).

Characterization of bacterial communities by cultivation would greatly underestimate the actual numbers and the diversity of the bacteria, because most drinking water bacteria cannot be cultured with standard methods (6). Therefore, cultivation-independent methods have been developed using 16S rRNA gene based approaches to identify bacterial species and assess their abundances within the community. These approaches include fluorescence in situ hybridisation (FISH), to detect specific bacteria in fixed samples, as well as clone libraries or fingerprints, to gain information about the overall structure and taxonomic composition of the analysed community. Fingerprints can describe structural features such as the relative abundance of a single species, the richness and the evenness of a community (6). Each bacterial

cell possesses only limited numbers of chromosomes, and therefore only a limited number of 16S rRNA genes. Hence, DNA-based techniques targeting 16S rRNA genes can be used to assess information about the presence and the relative abundance of single phylotypes. This allows calculating community measures such as richness and other diversity indices. In contrast, 16S rRNA concentration is dependent on the ribosome content of the bacterial cell, which rises with increasing growth rate or activity, and is currently understood to be a measure for bacterial activity (36). Therefore, using RNA-based fingerprints enables to screen for active phylotypes or to detect low abundant but active phylotypes that are not detected by DNA-based techniques (11, 22, 24, 28).

The composition of bacterial bulk water communities has been investigated several times, mostly with molecular identification of the present bacteria at the phylum or class level (4, 11). In most studies gram-negative bacteria such as *Alpha-*, *Beta-* and *Gammaproteobacteria* and *Bacteroidetes,* were the most abundant bacteria, but also high numbers of gram-positive bacteria like *Actinobacteria* were found in bulk water of DWSS. Only few studies identified bacteria to the species level and included not only DNA-based but also RNA-based community analyses (11, 37). RNA-based analyses enabled to detect additional phyla such as *Planctomycetes*, *Cyanobacteria*, *Acidobacteria,* and *Nitrospira* (11, 37).

The present study aimed at understanding the community structure and composition of the bacteria in drinking water biofilms, assess their state of activity and determine the effect of different tubing material. To this end, single-strand conformation polymorphism (SSCP) fingerprinting based on extracted DNA and RNA was applied to the amplified 16S rRNA genes followed by sequencing of major bands to quantify and identify members of the respective communities (41). Most DWSS in urban areas are older than 10 years, thus we concentrated on an old, well established network instead on a model system. The biofilm was sampled from different pipe materials at different locations in a small scale network, which has been built more than 20 years ago at the campus of the Helmholtz Centre for Infection research (HZI, Figure 4.1). Therefore, we assumed the sampled biofilms had reached a mature state in this network which is connected to the well studied DWSS of the Harzwasserwerke in Braunschweig, Germany (11, 18, 22). Bulk water was sampled at different sites in the HZI network and two other sites in the inner city of Braunschweig, to assess the community composition of the drinking water and the accompanying biofilm across the city.

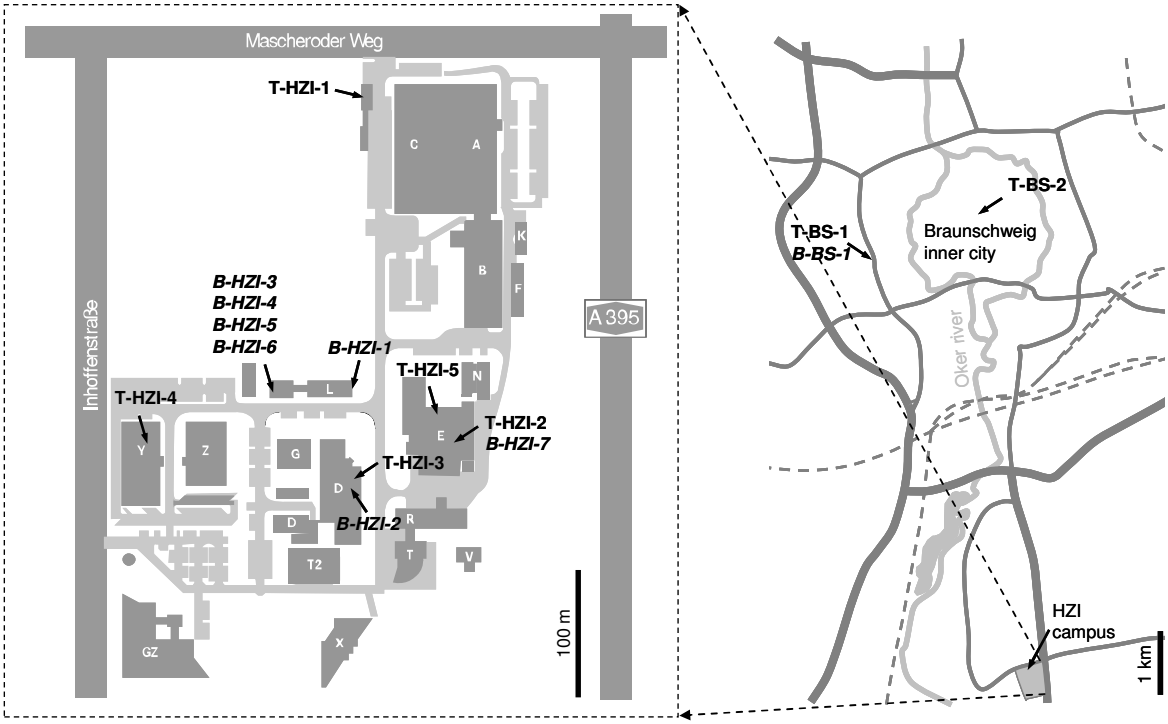


Figure 4.1: Sampling locations in the Braunschweig area and at the campus of the Helmholtz Centre of Infection research (HZI). Samples designated in bold italic are biofilm samples (B-X), samples only in bold are bulk water samples (T-X).

4.3 Material and Methods

4.3.1 Study sites and sampling.

Bulk water was sampled on June, 23 and 24, 2009 from several taps distributed on the campus of the Helmholtz Centre for Infection Research (HZI), Braunschweig-Stöckheim, Germany (T-HZI-1 to T-HZI-5) and from 2 households of the inner city of Braunschweig (T-BS-1, T-BS-2) (Figure 4.1, Table 1). Biofilm samples (B-HZI-2, B-BS-1) were taken in parallel to the bulk water sampling. Additional biofilm samples (B-HZI-1, B-HZI-3 to B-HZI-6) were obtained on May 7 and 14, 2009 when the L-building at the HZI campus was dismantled. (Figure 4.1, Table 4.2). All bulk water or biofilm samples were sampled in three different water networks connected to the HZI campus: (i) Municipal water network: a network supplying the whole city of Braunschweig including the HZI campus. (ii) Main network at the HZI campus: a circular network, supplying most of the buildings on the HZI campus. It is connected to the municipal water including pressure reducer, filter, and water thermostat. (iii) Looped fire water mains: network that supports fire hydrants and a few buildings on the HZI campus. It is connected directly to the municipal water. The drinking water originated from two surface water reservoirs (oligotrophic and dystrophic water) situated in a mountain range 40 km south of Braunschweig. Processing of the drinking water by the local supplier Harzwasserwerke GmbH included flocculation/coagulation, sand filtration and chlorination (0.2 - 0.7 mg l^{-1}). More details on the respective drinking water supply system are given elsewhere (11).

Drinking water microorganisms were sampled by filtration according to Eichler et al. (11). In brief, 5 liters of drinking water were filtered through a filter sandwich consisting of a 0.2 µm pore size polycarbonate filter (90 mm diameter; Nucleopore; Whatman, Maidstone, United Kingdom) with a precombusted glass fiber filter on top (90 mm diameter; GF/F; Whatman). Biomass harvested on filter sandwiches was stored at -70°C until further analysis. In parallel, heterotrophic plate counts (HPC) and direct counts were performed and relevant drinking water parameters, such as pH, conductivity, temperature, and chlorine concentration were determined (Table 4.1). Using sterile swabs (Heinz Herenz, Hamburg, Germany), drinking water biofilms were wiped off the wet surfaces of the tubing of different sampling locations and different materials. Swab heads with biofilm material were stored in 1.5ml reaction tubes at -70°C until further analysis (Table 4.2).

Table 4.1: List of bulk water samples and their main properties. No chlorine residues were detected in all samples. Values declared as "n.d." were not determined.

Desig-nation	Sampling location	Sampling Date	Sample origin	Water circulation	CFU/ml (R2A, 20°C, 48h)	CFU/ml (R2A, 36°C, 48h)	CFU/ml (R2A, 20°C, 72h)	CFU/ml (R2A, 36°C, 72h)	direct counts/ml [10^5 cells]	temperature [°C]	pH	conductivity (µS)
T-HZI-1	HZI M-build.	June 23, 2009	tap	municipal water	2.3	1.0	10.0	10.7	1.25	13.1	8.7	155
T-HZI-2	HZI E-build.	June 23, 2009	receiver tank	municipal water	0.3	5.7	0.3	5.7	2.35	14.5	8.6	157
T-HZI-3	HZI D-build.	June 23, 2009	room D0.04 tap	main circulation	0.3	0.0	0.7	0.0	2.57	12.5	8.5	158
T-HZI-4	HZI Y-build.	June 23, 2009	room Y4.19 tap	main circulation	n.d.	17.0	n.d.	17.0	1.95	20.8	8.2	156
T-HZI-5	HZI E-build.	June 23, 2009	reverse osmosis concentrate	main circulation	8.0	18.7	35.7	24.7	1.42	16.3	8.2	715
T-BS-1	Inner city of Braun-schweig	June 24, 2009	tap	municipal water	6.7	30.3	n.d.	n.d.	2.54	23.7	8.3	128
T-BS-2	Inner city of Braun-schweig	June 24, 2009	tap	municipal water	2.0	556.7	n.d.	n.d.	2.00	15.6	8.3	164

Table 4.2: Overview about the biofilm samples and their main properties.

Designation	Sampling location	Sampling Date	Sample origin	Material	Water circulation	Colour	Age (years)
B-HZI-1	HZI L-build.	May 7, 2009	prefilter	PVC	main circulation	brown	>20
B-HZI-2	HZI D-build.	June 24, 2009	tube at the tap	teflon®	main circulation	yellow beige	>7
B-HZI-3	HZI L-build.	May 14, 2009	main tube	copper	looped fire water mains	green	>20
B-HZI-4	HZI L-build.	May 14, 2009	water meter	stainless steel	looped fire water mains	brown	>20
B-HZI-5	HZI L-build.	May 14, 2009	control window	stainless steel	looped fire water mains	brown	>20
B-HZI-6	HZI L-build.	May 14, 2009	control window	glass	looped fire water mains	brown	>20
B-HZI-7	HZI M-build.	June 23, 2009	receiver tank	stainless steel	municipal water	brown	unknown
B-BS-1	Inner city of Braunschweig	June 24, 2009	flush water container	PVC	municipal water	brown	3

Table 4.3: Mean values of community structure indices for bulk water and biofilm communities calculated from relative abundance data of the SSCP fingerprints. Coefficient of variation is given in percent. [a] Values directly retrieved from raw data. [b] Values calculated from derived data.

Community	[a]Richness	[b]Margalef d	[b]Fisher's α	[b]Shannon H'	[b]Pielou eveness J'	[b]Simpson 1-λ
Bulk water (DNA)	64 ± 10%	13.72 ± 10%	80.73 ± 28%	3.45 ± 4%	0.83 ± 2%	0.97 ± 1%
Bulk water (RNA)	60 ± 6%	12.86 ± 7%	64.97 ± 19%	2.98 ± 8%	0.73 ± 9%	0.91 ± 6%
Biofilm (DNA)	54 ± 21%	11.40 ± 22%	53.33 ± 64%	2.94 ± 21%	0.74 ± 18%	0.89 ± 12%
Biofilm (RNA)	54 ± 28%	11.45 ± 28%	59.30 ± 80%	3.23 ± 14%	0.81 ± 8%	0.94 ± 4%

4.3.2 Heterotrophic plate counts and direct counts of drinking water bacteria.

HPCs were done in triplicates using an aliquot of the drinking water and the spread plate technique on R2A agar (Oxoid) plates. Incubation was carried out at two different temperatures according to the German drinking water ordinance (36°C for 48h and 22°C for 72h).

For total bacterial cell counts, formaldehyde-fixed samples (2% final concentration) were stained with Sybr Green I dye (1:10000 final dilution; Molecular Probes, Invitrogen) for 15min at room temperature in the dark. Five ml were filtered onto 0.2 µm pore size Anodisc filters (Whatman) and mounted with Citifluor on microscopic glass slides according to Weinbauer et al. (44). Slides were either analyzed directly with epifluorescence microscopy or stored frozen (-20°C) until examination. For epifluorescence microscopy, a microscope (Axioplan, Zeiss) with suitable fluorescence filters was used and the slides were examined using 100fold magnification. For each sample, 10 micrographs were taken and image sections of defined size (0.642mm x 0.483mm) were analyzed using the Image J software with the plug-in collection from MacBiophotonics (http://www.macbiophotonics.ca/). Typically, 500-800 bacterial cells per image were counted.

4.3.3 Nucleic acid extraction from drinking water and biofilms.

Bulk water DNA and RNA were extracted from the filter sandwiches. For extraction of DNA and RNA, a modified DNeasy/RNeasy protocol (Qiagen, Hilden, Germany) was used. In this procedure, sandwich filters were cut into pieces, incubated with lysis buffer containing 10mg/ml lysozyme (Sigma) for 60 min at 37°C (DNA) or 20 min at 20°C (RNA). After a proteinase K digestion (DNA) according to the manufacturer's instructions, the samples were heated to 70°C in a water bath for 20 min (DNA) or 15 min (RNA). After filtration through a polyamide mesh with 250 µm mesh size, absolute ethanol was added to the filtrate (ratio filtrate/ethanol 2:1) and the mixture was applied to the adequate spin-column of the Qiagen kit. From now on, the washing and elution protocol was followed according to the manufacturer's instructions.

Biofilm swabs were incubated with 220µl lysis buffer (2x TE) containing 10mg/ml lysozyme (Sigma) and 15 mg/ml proteinase K (Qiagen) for 20 min at 37°C. 350µl AL-buffer (DNeasy kit) or 700µl RLT-buffer (RNeasy kit) were added to the swab, both

supplied with corresponding extraction kit, followed by incubation for 5 min at 70°C. The lysate was removed from the swab by a short spin down and absolute ethanol was added to the lysate (ratio lysate/ethanol 2:1). The mixture was applied to the adequate spin-column of the kit. After this step, the protocol was followed according to the manufacturer's instructions.

For bulk water or biofilm RNA, a subsequent on-column DNase digestion (RNase-Free DNase Set, Qiagen, Hilden, Germany) was applied. Nucleic acids were eluted from the columns with DNase/RNase free water and stored at -20°C. The nucleic acids were quantified using Ribogreen (RNA or ssDNA quantification, Molecular Probes; Invitrogen) or Picogreen (dsDNA quantification, Molecular Probes; Invitrogen) according to Weinbauer and Höfle (44).

4.3.4 16S rRNA and 16S rRNA gene based community fingerprints.

PCR amplification of 16S rRNA genes from the extracted nucleic acids were performed using the previously described primers COM1 (5´-CAGCAGCCGCGGTAATAC-3´) and COM2 (5´-CCGTCAATTCCTTTGAGTTT-3´), amplifying positions 519 to 926 of the *Escherichia coli* numbering of the 16S rRNA gene (41). For single strand separation a 5´-biotin-labeled forward primer was used according to Eichler et al. (11). From 16S rRNA, reverse transcription was carried out before PCR using the First strand cDNA synthesis Kit (Fermentas, Canada) following the manufacturer's instructions with the same com-primers. PCR was carried out using 2 ng DNA/cDNA template in a final volume of 50 µl, starting with an initial denaturation for 15 min at 95°C. A total of 30 cycles (30s at 95°C, 30s at 55°C, and 1 min at 72°C) was followed by a final elongation for 10 min at 72°C. Amplification was achieved using HotStarTaq DNA polymerase (Qiagen, Hilden, Germany).

For the preparation of ssDNA and community fingerprints, the protocol described by Eichler et al. (11) was slightly modified. Briefly, magnetic streptavidin coated beads (Promega, Madison, Wis.) were applied to obtain ssDNA from the PCR amplicons. Quantification of the obtained ssDNA was performed on a 1.5% agarose gel by comparison with a low-molecular-weight marker (Invitrogen low-DNA-mass ladder). For SSCP fingerprint analysis, 25 ng of the obtained ssDNA was mixed with gel loading buffer (95% formamide, 10 mM NaOH, 0.25% bromphenol blue, 0.25% xylene cyanol) in a final volume of 7 µl. After incubation for 3 min at 95°C, the ssDNA samples were cooled on ice, loaded onto a nondenaturing polyacrylamide-like gel (0.6x

MDE gel solution; Cambrex BioScience, Rockland, Maine) and electrophoretically separated at 20°C at 400 V for 18 h on a Macrophor sequencing apparatus (Pharmacia Biotech, Germany). The gel was silver stained according to the method described by Bassam et al. (2). Dried SSCP gels were digitized using an Epson Expression 1600 Pro scanner, bands with an intensity of >0.1% of the total lane were considered for further statistical analysis. Similarity coefficients were calculated using Dice algorithm. Dendrograms were constructed with the Neighbor-Joining algorithm using the GelCompare II software (Applied Maths, Kortrijk, Belgium). Community indices were calculated using the software Primer 6 (PRIMER-E Ltd, Ivybridge, UK).

Sequence information from the single bands of the SSCP fingerprints was obtained following the protocol of Eichler et al.(11). Briefly, ssDNA bands were excised from the SSCP acrylamide gels, and boiled in Extraction buffer (10 mM Tris-HCl, 5 mM MgCl2, 5 mM KCl, 0.1% Triton X-100, pH 9). 7µl of the extraction solution was used in a reamplification PCR with the unbiotinylated COM primers described above. These amplicons were purified (MinElute kit, Qiagen, Hilden, Germany) and subsequently sequenced by cycle sequencing (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit; Applied Biosystems, Foster City, Calif.). Before analysis on an ABI Prism 3100 Genetic Analyzer, the products were purified using the BigDye Terminator purification kit (QIAGEN). Phylogenetic identification of the sequences was done either by the NCBI Tool BLAST/blastn (1) for comparison with the closest 16S rRNA gene sequence and for the identification of the closest described relative or the Ribosomal Data Base Project Seqmatch and Classifier tool (8, 43) for determination of corresponding phyla (RDP Release 10, Update 18, Jan 25, 2010). When more than two definite base pair differences existed in comparison with other phylotypes, we defined a new phylotype.

4.3.5 Accession numbers.

The partial 16S rRNA gene sequences retrieved from the fingerprints are accessible at the GenBank/EMBL/DDBJ accession numbers FR796543 to FR796698.

4.4 Results

4.4.1 General properties of bulk water and biofilm.

For all bulk water samples total bacterial cell counts, CFU on R2A agar and a set of physical and chemical parameters were determined (Table 4.1). For the sampling period, no chlorine was detected and the temperature varied between 12.5°C and 23.7°C. Heterotrophic plate counts (HPC) varied between 0.3 CFU/ml and 556.3 CFU/ml, while direct counts remained rather constant between 1.25 X 10^5 to 2.57 X 10^5 cells/ml. The pH value was always around 8.4 and the conductivity ranged from 128µS/cm to 164µS/cm, with the exception of the reverse osmosis concentrate sample (T-HZI-5), showing a conductivity of 715µS/cm. Biofilm samples differed in consistency and colour (Table 4.2). Some samples were slimy and yellow-beige (B-HZI-2), some were more friable and green (B-HZI-3). Most biofilm samples showed various orange-brown colours with a friable consistence.

4.4.2 Comparison of bacterial community structure in bulk water and biofilm using DNA-based SSCP fingerprints.

DNA-based SSCP fingerprints were used to analyse the community structure of bulk water and biofilm obtained from different sampling sites at the HZI campus and the inner city of Braunschweig (Figure 4.2a). For all five bulk water fingerprints no significant differences could be observed, except two additional intense bands in sample T-BS-2 which was sampled in the inner city of Braunschweig. In contrast, biofilm fingerprints sampled at different sampling sites were very diverse. Each biofilm fingerprint showed a unique pattern with sometimes only a few dominating bands. Comparative cluster analysis of bulk water and biofilm fingerprints confirmed the finding that the bulk water fingerprints were very similar to each other, while the biofilm fingerprints were very diverse (Figure 4.2b). All bulk water fingerprints clustered closely together, especially those originating from sampling sites that were located at the HZI campus had similarities higher than 85%. In contrast, biofilm fingerprints clustered together in two subclusters showing a high diversity with a maximum similarity of 40% to each other.

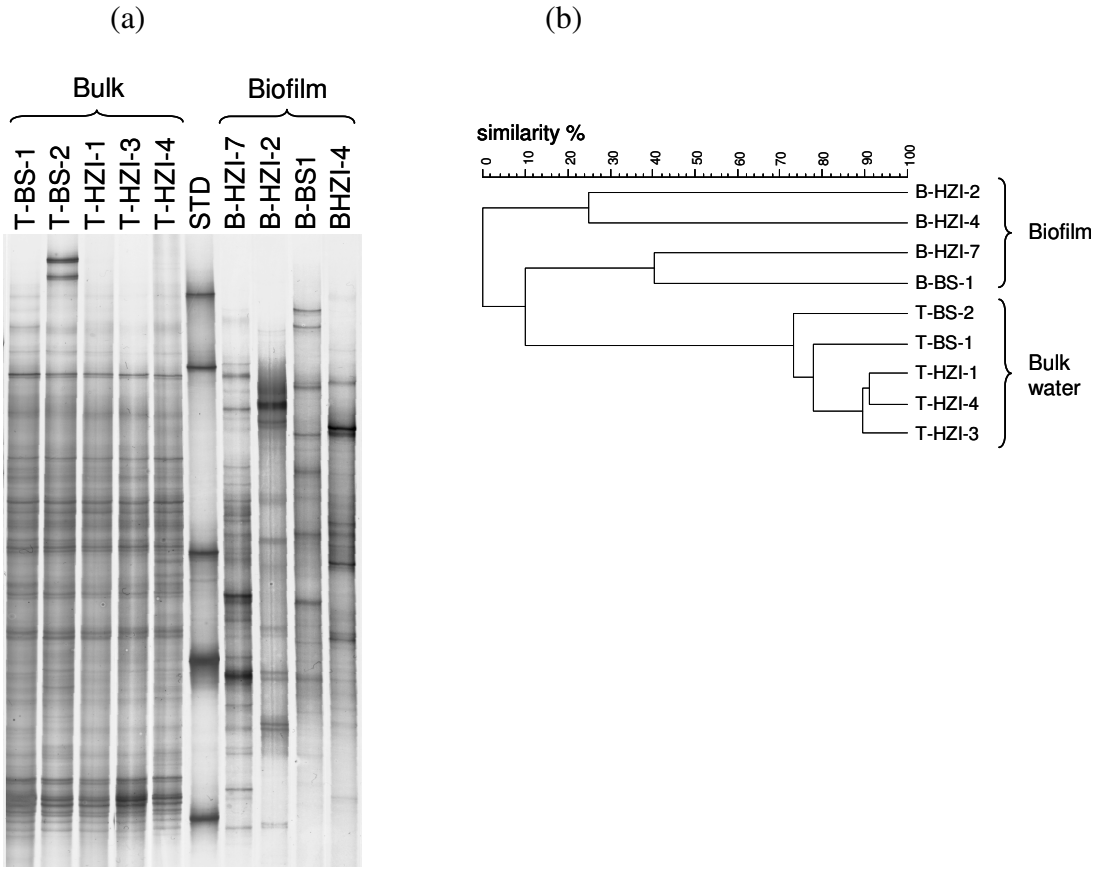


Figure 4.2: (a) Comparison of 16S rRNA gene based SSCP fingerprints of bulk water (left side, Bulk) and drinking water biofilm samples (right side, Biofilm). STD, standards. (b) Comparative cluster analysis of 16S rRNA gene based SSCP fingerprints of bulk water and biofilms using Pearson algorithm.

4.4.3 Comparison of DNA-based and RNA-based SSCP fingerprints of bulk water samples.

To compare the present bacterial microbiota with the active ones in bulk water, we used DNA-based and RNA-based 16S rRNA (gene) fingerprints, focusing on the five samples collected in the HZI small scale network (Figure 4.3a). The banding patterns of the DNA-based fingerprints were constant. The banding pattern of RNA-based fingerprints was also constant, but it was substantially different from DNA-based pattern. Only a few bands showed similar running distances and intensities in both DNA and RNA-based fingerprints, whereas most bands had different running distances. Accordingly, comparative cluster analysis of these fingerprints resulted in two clearly separated clusters, one with DNA-based fingerprints and the other with RNA-based fingerprints (Figure 4.3b).

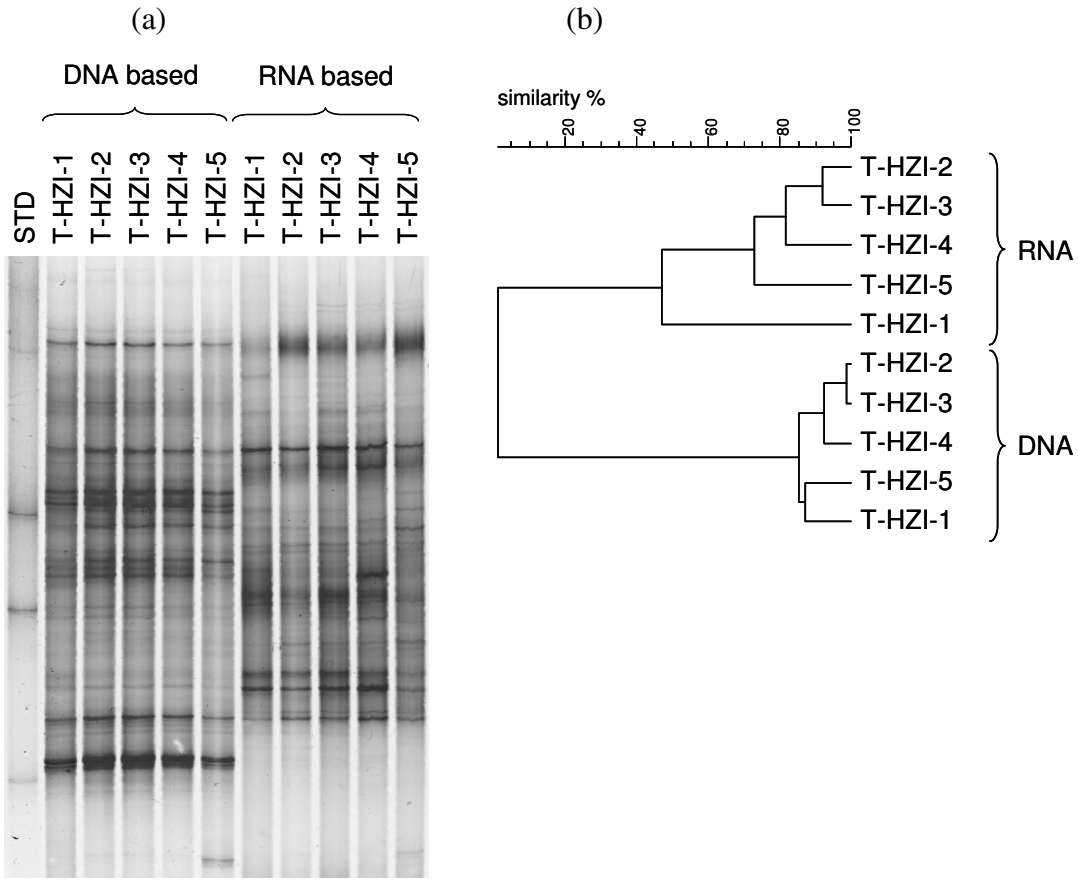


Figure 4.3: (a) 16S rRNA gene based SSCP fingerprints of bulk water (left side, DNA based) and 16S rRNA-based SSCP fingerprints of bulk water (right side RNA based). (b) Comparative cluster analysis of DNA and RNA-based SSCP fingerprints of bulk water using Pearson algorithm.

DNA-based fingerprints, sampled only at the HZI campus, clustered very closely with similarities above 80%. RNA-based fingerprints showed similar subclusters with high similarities between bulk water sample T-HZI-2, T-HZI-3 and T-HZI-4 and lower similarity for the samples T-HZI-1 and T-HZI-5. Interestingly, the sample T-HZI-1, representing the municipal water when it enters the small HZI network, had the most different RNA-based banding pattern.

4.4.4 Comparison of DNA-based and RNA-based SSCP fingerprints of biofilm samples.

Comparison of SSCP fingerprints based on the analysis of 16S rRNA and 16S rRNA genes was also done for eight biofilm samples, seven of them were from the HZI campus and one biofilm (B-BS-1) was obtained in the inner city of Braunschweig (Figure 4.4a). The high variation of biofilm fingerprints obtained from different sampling sites was obvious for DNA and RNA-based fingerprints. However, similar banding patterns were observed in biofilms sampled at adjacent sampling sites irrespective of the tubing material, i. e. samples from the glass surface (B-HZI-6) and from the steel filter grid of the control window (B-HZI-5). These similar banding patterns were found for both types of fingerprints. In contrast to the bulk water fingerprints, the DNA-based biofilm fingerprints looked more similar to the RNA-based fingerprints of the same sample. Comparative cluster analysis of the biofilm samples showed similarities clearly lower (range 5% - 60%) than those obtained for bulk water samples, confirming the higher variability of the biofilm communities (Figure 4.4b). Mostly, DNA-based and RNA-based fingerprints of the same sample clustered together and formed often a DNA-RNA subcluster with similarities ranging from 25% to 40% (see B-HZI-5, B-HZI-6, B-HZI-3, B-HZI-1, B-HZI-7, and B-BS-1 in Fig 4b). Additionally, the fingerprints of biofilms collected in the looped fire water mains (B-HZI-3 - B-HZI-6), a circular pipeline, formed a separate subcluster with similarities between 25% and 60%. The other biofilm fingerprints formed several subclusters. The subcluster of B-HZI-7 (steel) and B-BS-1 (plastics, PVC) with similarities between 30% and 45% contained fingerprints of biofilms that both had direct contact with the municipal water. The B-BS-1 flush water container was located in the inner city of Braunschweig and was therefore directly supplied with municipal water, while the B-HZI-7 receiver tank was the first tank in the HZI campus, where the municipal water was collected to reduce the municipal water pressure. Overall, fingerprints of biofilms with physically related sampling sites were more similar to each other than fingerprints grown on the same material but on physically unrelated sampling sites.

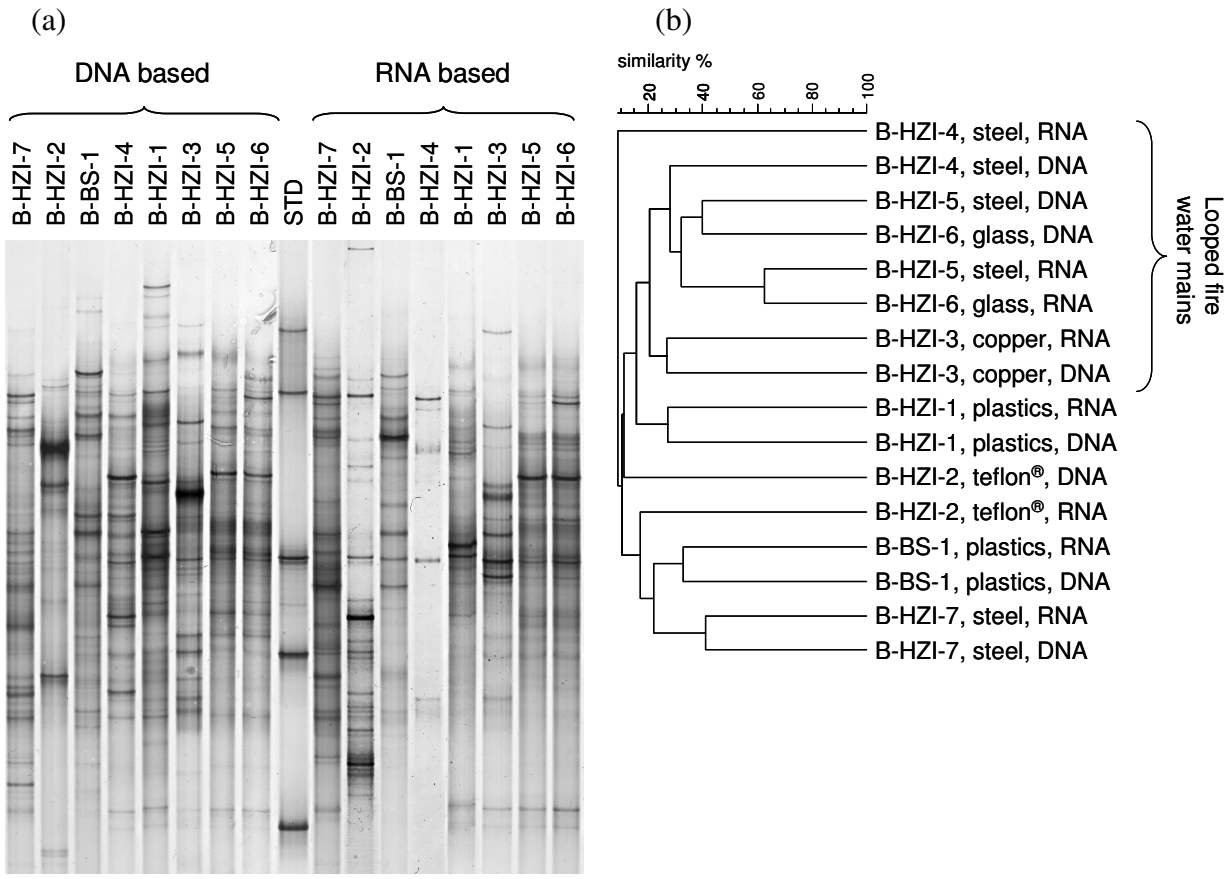


Figure 4.4: (a) 16S rRNA gene based SSCP fingerprints of biofilm samples (left side, DNA based) and 16S rRNA-based SSCP fingerprints of biofilm (right side, RNA based). (b) Comparative cluster analysis of DNA and RNA-based SSCP fingerprints of the biofilm samples.

4.4.5 Comparison of bulk water and biofilm community structure.

We calculated rank-abundance curves from the DNA-based fingerprints of the bulk water and biofilm samples to compare their overall community structure (Figure 4.5). These rank abundance curves were based on the assumption that each band of a fingerprint represents a single phylotype and the band intensity is proportional to its relative abundance. Clear differences in these rank abundance plots in the community structure of bulk water and biofilm became apparent. In the bulk water communities between 52 (T-BS-2) and 69 (T-HZI-1) phylotypes were found above the detection limit of relative abundance of 0.1 %. The relative abundance of the most abundant phylotype, i.e. rank one, ranged from 6 % in sample T-HZI-1 up to 15 % in sample T-BS-1. Although biofilm fingerprints differed strongly, their rank abundance curves were quite similar. The biofilm samples had only 30 (B-HZI-2) to 47 phylotypes (B-BS-1) above

the detection limit. The most abundant phylotypes reached abundances between 13% (B-BS-1) and 30% (B-HZI-2). Exponential regression analysis of all biofilm and bulk water values and subsequent semi-logarithmic plot revealed significantly different slopes and intersections (Figure 4.5 insert). The x-axis intercepts, where the curve reaches the detection limit, are an estimator of the richness of bacterial communities. In bulk water this value was 65.0, whereas in biofilm this value was 47.3, i. e. the richness of biofilm communities is reduced by more than a quarter compared to bulk water.

To understand in detail the differences in the community structure of bulk water and biofilm samples, six different indices were calculated (Table 3) for both types of fingerprints. Relative abundance data of the fingerprints were used for these calculations, assuming that each band in a fingerprint represents one species. As we used relative abundance data, all abundances add up to 100% and the diversity indices were calculated using proportions of each species instead of absolute numbers of individuals. The indices Margalef d and Fisher's α are measures for richness, while the Shannon H', the Pilou evenness J', and the Simpson 1-λ are measures for diversity, dominance and equitability, respectively. In general, higher Margalef d and Fisher's α were obtained for bulk water communities than for biofilm communities on both types of fingerprints. In the biofilm community a higher standard deviation was observed. The Pilou evenness J' and the Simpson 1-λ showed similar values for bulk water communities in the DNA-based data and biofilm communities in the RNA-based data, which were always a bit higher than the indices for bulk water communities in the RNA-based data and biofilm communities in the DNA-based data. Overall, all biofilm communities showed higher relative abundances of single phylotypes with a reduced richness, i.e. the total number of all phylotypes above the detection limit, compared to bulk water communities.

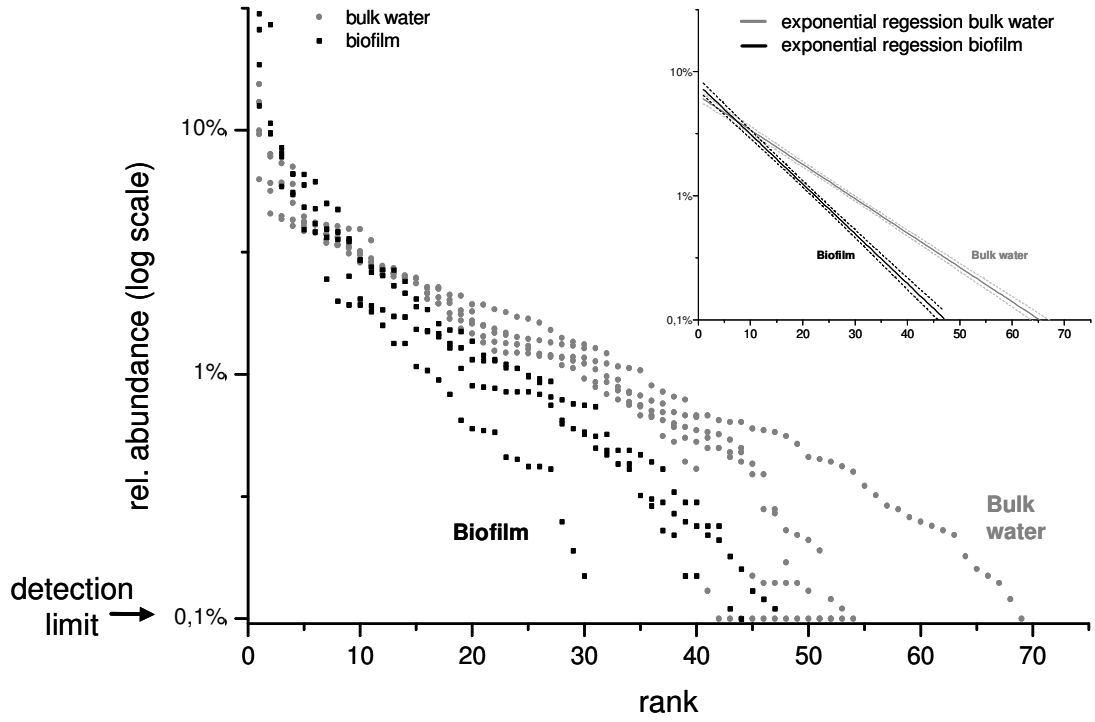


Figure 4.5: Rank abundance plot of bulk water (gray dots) and biofilm samples (black squares) using relative band intensities from DNA-based fingerprints as a measure for relative abundance. Insert shows the regression analysis of plotted rank abundance curves. Dotted lines indicate 95% confidence band. Bulk water: y = 5.8691(-0.045015x); R² = 0.885. Biofilm: y = 7.9153(-0.092472x); R² = 0.898.

4.4.6 Taxonomic composition of bulk water communities.

Excising and sequencing of the major SSCP-bands, approximately all bands above 1% relative band intensity, of bulk water fingerprints and subsequent alignment of the obtained sequences resulted in a set of 44 unique phylotypes. 26 phylotypes were obtained from DNA-based fingerprints, and 18 phylotypes from RNA-based fingerprints, with no identical phylotypes among DNA-based and RNA-based sequences. Phylogenetic identification of the phylotypes is summarized in Supplementary Table S1. We used a sequence similarity of 90% or higher for the 16S rRNA gene to rate the phylotype as of "aquatic origin". 16S rRNA gene sequence similarities below 90% were regarded as too low to give information on the potential habitat of a phylotype. Based on these criteria, 75% of the bulk water phylotypes were considered as of aquatic origin, with most of them from freshwater habitats. The observed phylotypes (PTs) were mainly related to members of taxonomic groups typical for freshwater according to Zwart et al. (46), such as *Bacteroidetes* (11 PTs, 25%),

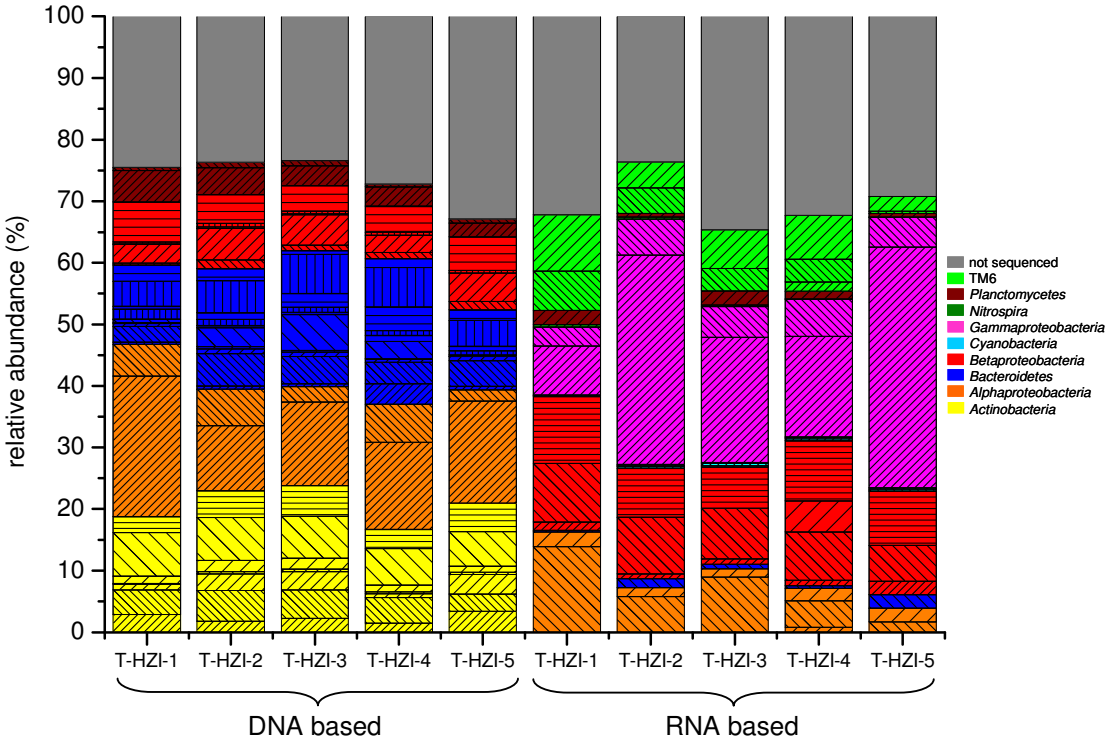
Betaproteobacteria (9 PTs, 20%), *Actinobacteria* (7 PTs, 16%), and *Alphaproteobacteria* (5 PTs, 11%). A low number of phylotypes were observed for members of *Cyanobacteria*, *Nitrospira*, *Planctomycetes*, *Gammaproteobacteria* and the candidate division TM6 (Supplementary Table S3).

The taxonomic composition of the bacteria from bulk water is given in Figure 4.6a using relative abundances estimated from band intensities. For the bulk water, on average 72% (coefficient of variation c_v: $\pm$6.2%) of the bands could be assigned to a specific phylotype. The taxonomic compositions of all bulk water samples was very similar to each other, but with no overlap between DNA- and RNA-based fingerprints. DNA-based fingerprints were mainly composed of members of *Bacteroidetes* (12% - 20% sum of relative abundances), *Actinobacteria* (17% - 22%), and *Alphaproteobacteria* (18% - 27%), while the former two phyla were almost exclusively detected in DNA-based fingerprints (Figure 4.6a). RNA-based phylotypes belonged mainly to *Betaproteobacteria* (17% - 23%), *Gammaproteobacteria* (10% - 35%), and candidate division TM6 (3% - 16%), whereas the latter two phyla were only present on the RNA-based fingerprints. Though only two gammaproteobacterial phylotypes were found, these phylotypes were the most abundant in RNA-based fingerprints.

4.4.7 Taxonomic composition of biofilm communities.

A set of 112 unique phylotypes was obtained from sequencing of all major SSCP bands from both types of fingerprints from four physically unrelated biofilm samples (supplementary Table S2). We could resolve, at best, the species level using the about 400nt long sequences obtained from the fingerprints. This might be enough taxonomic resolution for environmental phylotypes that often have no closely related cultured neighbor species. 55 phylotypes occurred only in DNA-based fingerprints, and 44 phylotypes occurred only in RNA-based fingerprints, whereas 13 phylotypes were found in both types of fingerprints (supplementary Table S3, Figure 4.6b). Sequence comparison with all bulk water phylotypes revealed no congruence between phylotypes from bulk water and biofilm communities (Figure 4.7). Using the same criteria as for the bulk water samples, 17% of all biofilm phylotypes were considered to be of aquatic origin, 9% were considered to be of "biofilm origin" and 32% were considered to be of "soil, sludge or sediment origin". In case of aquatic origin, many of the phylotypes had highest similarities to phylotypes found in either in waste water or water treatment plants.

(a)


relative abundance (%)
100
90
80
70
60
50
40
30
20
10
0
T-HZI-1
T-HZI-2
T-HZI-3
T-HZI-4
T-HZI-5
T-HZI-1
T-HZI-2
T-HZI-3
T-HZI-4
T-HZI-5
DNA based
RNA based
not sequenced
TM6
Planctomycetes
Nitrospira
Gammaproteobacteria
Cyanobacteria
Betaproteobacteria
Bacteroidetes
Alphaproteobacteria
Actinobacteria

(b)

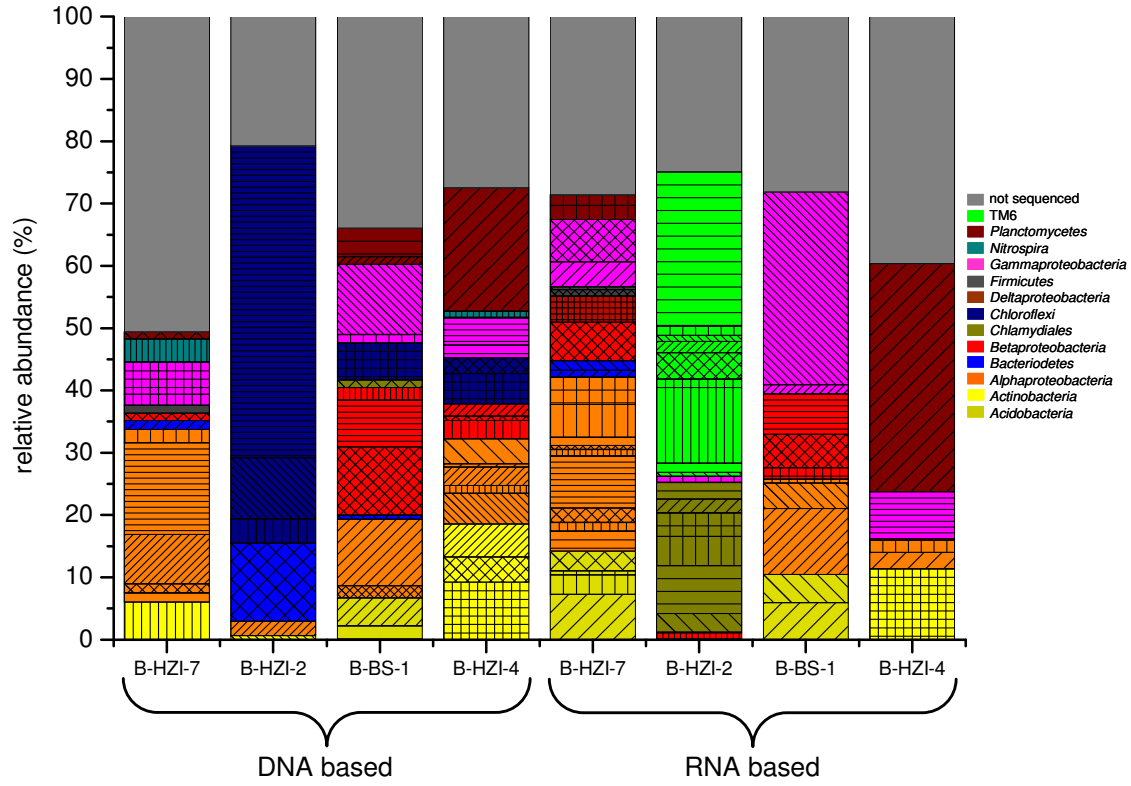

relative abundance (%)
100
90
80
70
60
50
40
30
20
10
0
B-HZI-7
B-HZI-2
B-BS-1
B-HZI-4
B-HZI-7
B-HZI-2
B-BS-1
B-HZI-4
DNA based
RNA based
not sequenced
TM6
Planctomycetes
Nitrospira
Gammaproteobacteria
Firmicutes
Deltaproteobacteria
Chloroflexi
Chlamydiales
Betaproteobacteria
Bacteriodetes
Alphaproteobacteria
Actinobacteria
Acidobacteria

Figure 4.6: (a) Comparison of relative abundances of the major phylotypes (relative abundances above 1%) found in the bulk water communities. Left part represents the phylotypes from the DNA-based SSCP fingerprints. Right part represents the phylotypes from the RNA-based SSCP fingerprints. The colors are corresponding to the major phylogenetic groups of the phylotypes. The differently hatched parts of the stacked bars represent the single phylotypes identified. (b) Comparison of relative abundances of the major phylotypes found in four selected biofilm fingerprints. The biofilms were not directly physically related. Left part represents the phylotypes from the DNA-based SSCP fingerprints. Right part represents the phylotypes from the RNA-based SSCP fingerprints. The colors are corresponding to the major phylogenetic groups of the phylotypes. The differently hatched parts of the stacked bars represent the single phylotypes identified.

The relative abundances of the single phylotypes in the biofilm communities are compared in Figure 4.6b. Overall, each biofilm fingerprint represented an individual bacterial community with a unique taxonomic composition with some similarities between DNA- and RNA-based fingerprints. Most of the phylotypes belonged to the *Alphaproteobacteria* (28 PTs, 26%), followed by *Gammaproteobacteria* (12 PTs, 11%) and the candidate division TM6 (12 PTs, 11%). In medium numbers we found *Chlamydiales* (10 PTs, 9%) and *Betaproteobacteria* (10 PTs, 9%). Taxonomic groups with numbers of phylotypes lower than 10 were *Acidobacteria*, *Planctomycetes*, *Firmicutes*, *Actinobacteria*, *Chloroflexi*, *Bacteroidetes*, and *Nitrospira* (Supplementary Table S3). Phylotypes belonging to *Chlamydiales* and the candidate division TM6 were mainly found in sample B-HZI-2, which was sampled from a Teflon® tube attached to the tap. Furthermore, this biofilm was the only showing pronounced differences between the taxonomic composition of DNA-based and RNA-based fingerprints. In this biofilm community, based on DNA fingerprints, only three phylotypes belonging to the phylum *Chloroflexi* represented approximately two thirds of phylotypes, while in other biofilms, the *Chloroflexi* phylotypes accounted for a maximum abundance of only 7.4%. In the RNA-based fingerprints of this biofilm sample, a completely different set of phylotypes was observed. Here, 47% belonged to *Chlamydiae* phylotypes and 22% belonged to candidate division TM6 phylotypes. These phyla had only marginal abundances in the other three biofilm samples.

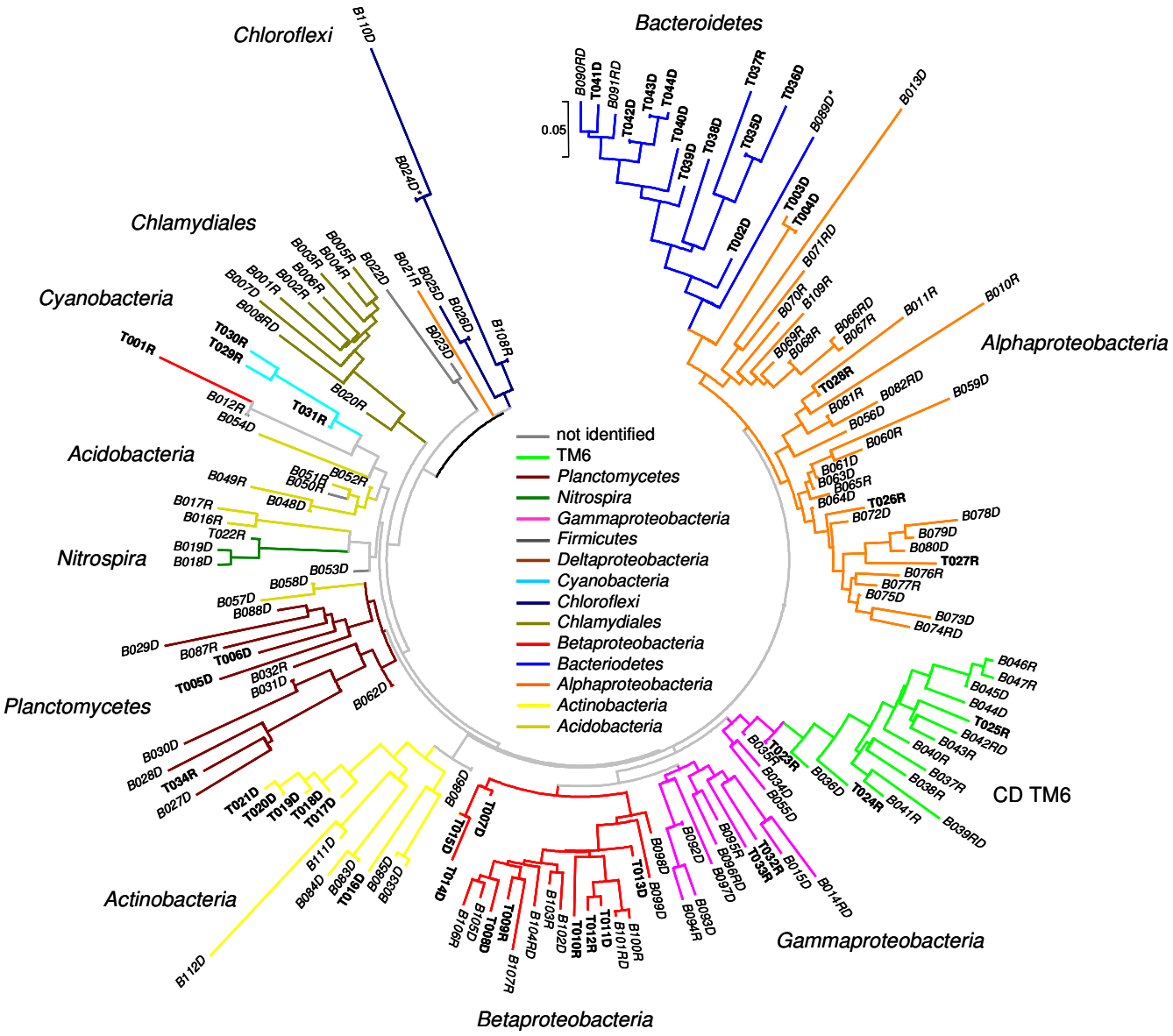


Figure 4.7: Phylogenetic tree of all 16S rRNA gene sequences obtained from bulk water and biofilm phylotypes (detailed lists of the single phylotypes are given in Supplementary Table 1 and 2). Designation of sequences B: biofilm phylotype; T: bulk water phylotype; D originated from DNA-based fingerprint; R: originated from RNA-based fingerprint. All bulk water phylotypes are indicated in bold. The taxonomic tree was inferred using the Neighbor-Joining method. Evolutionary distances were computed using the Maximum Composite Likelihood method.

4.5 Discussion

All measured physical and chemical bulk water parameters were similar to those monitored by the local drinking water supplier Harzwasserwerke (17) and were therefore considered to be typical for Braunschweig's drinking water. The discrepancy between CFU and direct counts of four to five orders of magnitude confirmed once again the detection gap between culturable and non-culturable drinking water bacteria (9) and emphasizes the need of culture-independent detection methods as used in this study. All bulk water parameters, with the exception of temperature, remained rather constant and were therefore, independent of the sampling site. In contrast, biofilm properties were very diverse. The friable consistence and the green-turquoise colour were indicators for biofilm grown on copper (B-HZI-3), probably due to a high copper compound content in the biofilm. Biofilms grown on steel (B-HZI-4, -5 and -7) tended to be friable and orange-brown, conceivably an indicator for ferric oxide. Biofilm sample B-HZI-6 showed the same consistence and colour as sample B-HZI-5, although it was sampled from a glass surface, but only approximately 4 cm from the sampling position of B-HZI-5. The slimy yellow appearance of biofilm sample B-HZI-2 indicated a completely different community composition from other biofilms due to different growth conditions. It established at room temperature and although the tap, where the tube was attached, was frequently used, the biofilm was often in contact with air, so that it maybe often dried out.

4.5.1 Comparison of bacterial community structure of bulk water and biofilms.

The high similarity of all bulk water fingerprints demonstrated the stability of the overall community structure of the drinking water bacteria with little spatial variation (Figure 4.3b). The drinking water community in this DWSS was independent from the sampling site, as confirmed by DNA- and RNA-based fingerprints. These findings were consistent with Eichler et al. (11), who sampled the same DWSS along the production line from the reservoirs providing the raw water and the different treatment steps to the tap. They showed, that after chlorination, the drinking water community was almost identical all along the DWSS. We observed clearly separated subclusters for DNA- and RNA-based fingerprints indicating large differences between the present and the active bacteria in bulk water communities. DNA-based fingerprints clustered closely together,

showing that the community of present bacteria was not affected by minor changes of the physico-chemical conditions of the bulk water indicating a high resilience of the drinking water community across the distribution network of the city of Braunschweig.

In contrast to bulk water, biofilm fingerprints showed large differences of the bacterial communities present (Figure 4.4a). Each biofilm showed a unique pattern of bands indicating that each biofilm consisted of a unique community. As the community structure is influenced by environmental conditions (42), each biofilm habitat seemed to have its own micro-environmental conditions like pH, oxygen concentration or nutrient availability, provided not only by surface material or water quality, but also by the community itself. These findings were confirmed by the comparative cluster analysis of all biofilm fingerprints (Figure 4.4b). In general, the clustering distance reflects the distance between sampling sites. All fingerprints of biofilms that were sampled in the looped fire water mains (B-HZI-3 to B-HZI-6) built their own subcluster, although these biofilms were grown on different surface material (copper, steel, and glass). This effect was especially apparent in the control window subcluster. Here, the similarity of adjacent sampling sites (B-HZI-5 and -6) was so high, that the RNA-based fingerprints of biofilms grown on steel or glass clustered even more closely together than their corresponding DNA-based fingerprints. Also, the fingerprints of biofilms grown in the municipal water (B-BS-1 and B-HZI-7) showed similarities despite different surface materials (steel and PVC). The observed similarity of physically related biofilms and the low dependency of the community structure on the surface material could be explained by the mutual influence of adjacent biofilm communities. Although the first colonisation of surfaces has been shown to be dependent on the surface material (10, 25), an adjacent coexistence for years may lead to mutual influence of biofilms by exchange of bacteria. It is conceivable, that once the surface is covered by a first, material specific biofilm, it is overgrown by a nearby biofilm community that is more independent from the surface material. From our observations, we hypothesize that during several years physically related biofilm communities will show similar community structures. Confirming our observation, Martiny et al. showed for their model DWSS that after three years most biofilms from different sampling positions clustered together and therefore possessed a homogeneous bacterial composition (30).

In general, DNA-based and RNA-based fingerprints of biofilms were much more similar to each other than those of bulk water fingerprints. In bulk water, a strong

impact from the source water bacteria was observed especially on the DNA-based fingerprints by Eichler et al. (11), whereas the RNA-based fingerprints were less related to the source water bacteria. This source water impact was not observed in biofilm fingerprints, i.e. the biofilm community does not reflect the drinking water community originating in the freshwater reservoirs of the Harz Mountains. The relatedness within the biofilm fingerprints suggests, that in biofilms those bacteria are growing that are also highly active, finally leading to higher abundances in the DNA-based fingerprints.

The source water influence on the bulk water community is also reflected in other community characteristics. All bulk water communities showed higher richness or indices that are estimates of richness, such as Margalef d and Fisher's α (13), than biofilm communities (Table 3). As the reservoirs are large freshwater environments with permanent exchange of biomass and bacteria, the source water influenced bulk water showed a high richness, also confirmed by Eichler et al. (11). In contrast, source water dependence of biofilms communities is rather negligible and the similarity between present and active bacteria is higher than in bulk water communities. We assume that only those bacteria were successful in colonizing biofilms that can actively contribute to the succession of the biofilm, while those bacteria that cannot fill perfectly the narrow niches in biofilms vanished after time. This process would lead to a lower richness in biofilm than in the corresponding bulk water. This assumption would also explain why we found lower richness values for biofilms than for bulk water, while Martiny et al. observed similar relative richness values for biofilm and the corresponding bulk water after three years of succession (30). In their observation, the richness of the biofilm showed a clear declining trend for the last year, which didn't reach a plateau until the end of their study. Biofilms investigated in our study were definitely older than three years, therefore we can assume that this trend of deminishing number of species continued to reach a lower richness than the corresponding bulk water.

The mean slope of rank abundance curves for DNA-based bulk water and biofilm fingerprints differed substantially, demonstrating considerable differences between both communities (Figure 4.5). This is reflected by significantly different richness values indicated by the y-axis intercept, as discussed above. For the bulk water, our rank abundance data suggest that there is a wide variety of low abundant phylotypes, which were at or below our detection limit of 0.1% relative abundance. This is consistent with

other studies of pelagic bacterial communities including drinking water (6, 11, 35). In contrast to the bulk water community, each biofilm community used for these calculations might consist, as demonstrated, of a unique set of bacteria. Nevertheless, all biofilm rank abundance curves showed the same trend for slope and axis interception. This behaviour suggests, with respect to the diversity and structure of biofilm communities that there are similar mechanisms structuring these communities in DWSS, i.e. all biofilms provide a similar number of niches but are filled with different species. The same overall structure for drinking water biofilms was also observed in a recent study using pyrosequencing (20), i. e. a rapid decrease of abundant species with a long "tail" of rare species. This study provided three times more phylotypes due to its substantially lower detection limit in comparison to SSCP fingerprints (14, 31).

4.5.2 Comparison of taxonomic composition of bulk water and biofilm communities.

More than 90% of all detected phylotypes in the bulk water had the highest 16S rRNA gene similarity to uncultured bacteria. This could explain the low CFU values in comparison to the total bacterial cell counts of the bulk water (Table 4.1). Almost all of these phylotypes were considered to be of aquatic origin and belonged to typical freshwater taxonomic groups (32, 46). Those bacteria originated mostly from the two source waters, the Grane reservoir and the Ecker reservoir, as shown by Eichler et al. (11). Most of the phylotypes were identical, or at least very similar, in the analysed 16S rRNA gene sequence to those formerly observed by Eichler et al. (11) and Kahlisch et al. (22). Although seasonal changes in the community structure were demonstrated during one year, the overall composition of the community in the DWDS remained rather constant for about four years (18). This is evidence that the concept of a stable "core community" is applying to bulk drinking water communities from man-made freshwater environments leading to good resilience to temporally and spatially limited disturbances of the bacterial community (19).

In contrast to bulk water, the majority of biofilm phylotypes were considered to be of soil, sludge or sediment origin or of biofilm origin. Members of the key genera *Rhizobiales*, *Nitrospira* or *Thiobacillus* which we found in drinking water biofilms, are known to contribute to the biogeochemical cycling of nitrogen or sulphur. Also many other uncultured bacteria with high similarities to denitrifying species were found in

these biofilms. This suggests that the species in biofilms form a system of complex interactions to build a community metabolism. Although each biofilm consists of a set of unique phylotypes, these phylotypes belonged to classes, which were present in most biofilms in comparable abundances, especially members of the *Alphaproteobacteria*, *Gammaproteobacteria*, and *Deltaproteobacteria* (Figure 4.6b, 4.7). This may indicate that different biofilm communities provide niches with similar conditions which are indeed filled by different species belonging to the same class.

In the biofilm samples B-HZI-2 and in B-BS-1 seven different uncultured phylotypes of the *Chlamydiales* with high 16S rRNA sequence similarity to members of the *Parachlamydiaceae* such as *Parachlamydia acanthamoebae*, *Protochlamydia naegleriophila*, and *Neochlamydia hartmannellae* were observed. *Parachlamydiaceae* are sometimes found in patients with community acquired pneumonia (29) and it is known that they are able to enter and replicate within human macrophages (16). Therefore, these species could represent emerging pathogens for human pneumonia or other respiratory diseases (15). More research is needed to investigate this potential health risk when these types of biofilms occur.

Bulk water community structure and biofilm community structure were characterized by large differences as observed with both types of fingerprints. These differences, already described in other studies (30), also applied for the respective community compositions. Not a single identical phylotype was detected in the planktonic bacteria of bulk water and the attached bacteria living in biofilm communities above the detection limit of 0.1% abundance. Thus, it can be inferred that no major exchange between the two core communities occurred. The current model of "abundant and rare members" describes the bacterioplankton community in pelagic ecosystems, consisting of a core community with few taxa that are highly abundant and a seed bank with nearly infinite numbers of very low abundant phylotypes (19, 21, 34). As a biofilm provides niches that differ from those in the bulk water, we hypothesize that the low abundant bacteria from the bulk water seed bank were recruited for the biofilm development and biofilm succession. This represents a possible mechanism for transition from a rare member of the bulk water community to an abundant member of the biofilm community.

A great discrepancy between DNA and RNA derived phylotypes was observed for bulk water with no overlap between them. This discrepancy was already found before in previous studies on the DWSS of Braunschweig, the two water reservoirs Grane

reservoir and Ecker reservoir, where the drinking water is originating, and other aquatic environments like the surface water in the Baltic Sea (5, 6, 11, 22). The two hypotheses given recently to explain this phenomenon were: i) highly active communities show less discrepancies between DNA- and RNA-based fingerprints and ii) reduced oxic stress in anaerobic environments result also in less discrepancies (5). Both explanations are conceivable from the mature drinking water biofilms studied.

Although species of the class *Gammaproteobacteria* or members of candidate division TM6 were dominant in RNA-based fingerprints of bulk water, those phylotypes could not be detected in DNA-based fingerprints. In bulk water, there were also high abundances of *Actinobacteria* detected in the DNA-based, but not in the RNA-based fingerprints. In biofilm sample B-HZI-2, which was a biofilm with temporarily contact to air, a great discrepancy between DNA and RNA-based phylotypes was observed, with *Bacteroidetes* and *Cyanobacteria* in the DNA-based fingerprints and members of the candidate division TM6 and *Chlamydiales* in the RNA-based fingerprints. *Bacteroidetes* and bacteria from the candidate division TM6 were also found in bulk water only in the DNA-based fingerprints or in the RNA-based fingerprints, respectively. This suggests that there are major differences in the content of ribosomes in a bacterial cell, so that bacteria with a high ribosomal content are highly overrepresented and bacteria with a low ribosome content are underrepresented in comparison to the DNA-based fingerprints. It seems that this described discrepancy is very strong effect leading to a intense overestimation of phylotypes so that other phylotypes, which are maybe present in both types of fingerprints are barely visible in faint bands, which we did not sequence in our study. Interestingly, this phenomenon was not observed in the other biofilm samples. In theses biofilms many phylotypes and nearly all phyla were present in both types of fingerprints. This suggests that there are still unknown environmental conditions, besides the activity of a bacterium, influencing the ribosome content of a cell leading to this DNA / RNA discrepancy when determining the community composition with either DNA- or RNA-based methods.

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Supplementary Table 4.1: Taxonomic identification of single phylotypes found in bulk water SSCP fingerprints shown in Figure 4.3a. Sample origin: D, only present in DNA-based fingerprints; R, only present in RNA-based fingerprints; DR, present in DNA and RNA-based fingerprints.

Phylotype designation	Sample origin	GenBank accession no.	Taxonomic group	Closest 16S rRNA gene sequence (Accession no.)	source of closest sequence	% Similarity	Closest described species (Accession no.)	% Similarity
T015	D	FR796669	Actinobacteria	Uncultured actinobacterium clone CB31D05 16S ribosomal RNA gene, partial sequence (EF471701)	whole surface water from Chesapeake bay	89%	Tetrasphaera veronensis (Y14596)	87%
T016	D	FR796670	Actinobacteria	Uncultured bacterium clone GC1m-4-84 16S ribosomal RNA gene, partial sequence (EU640899)	Lake Michigan	94%	Brevibacterium albus (EF158852)	86%
T017	D	FR796671	Actinobacteria	Uncultured bacterium isolate SSCP band GR1-D2-1_8 16S ribosomal RNA gene, partial sequence (DQ077614)	drinking water distribution system HWW	88%	Candidatus Planktophila limnetica (FJ428831)	91%
T018	D	FR796672	Actinobacteria	Uncultured actinobacterium clone I-DW-40 16S ribosomal RNA gene, partial sequence (GQ453106)	drinking water	94%	Candidatus Planktophila limnetica (FJ428831)	93%
T019	D	FR796673	Actinobacteria	Uncultured Actinomycetales bacterium clone Gap-2-37 16S ribosomal RNA gene, partial sequence (EU642138)	Milwaukee harbor	95%	Candidatus Planktophila limnetica (FJ428831)	93%
T020	D	FR796674	Actinobacteria	Uncultured actinobacterium clone TR1F1 16S ribosomal RNA gene, partial sequence (EU117957)	lake epilimnion	98%	Candidatus Planktophila limnetica (FJ428831)	96%
T021	D	FR796675	Actinobacteria	Uncultured bacterium clone CABC1F12 16S ribosomal RNA gene, partial sequence (GU127254)	aphotic layer; anoxic zone	94%	Candidatus Planktophila limnetica (FJ428831)	91%
T003	D	FR796657	Alphaproteobacteria	Uncultured bacterium isolate SSCP band DNA1-12-14 16S ribosomal RNA gene, partial sequence (DQ077623)	drinking water distribution system HWW	100%	Candidatus Pelagibacter ubique (EU410957)	86%

T004	D	FR796658	Alphaproteobacteria	Uncultured bacterium isolate SSCP band DNA1-12-14 16S ribosomal RNA gene, partial sequence (DQ077624)	drinking water distribution system HWW	98%	Ehrlichia ewingii (U96436)	84%
T026	R	FR796680	Alphaproteobacteria	Uncultured alpha proteobacterium clone AKYH1214 16S ribosomal RNA gene, partial sequence (AY921890)	farm soil adjacent to a silage storage	90%	Prosthecomicrobium consociatum (FJ560750)	90%
T027	R	FR796681	Alphaproteobacteria	Bosea sp. 7 GUW 16S ribosomal RNA gene, partial sequence (EU496542)	water; natural oil seeps	99%	Bosea eneae (EF519707)	99%
T028	R	FR796682	Alphaproteobacteria	Bosea sp. RA62 16S ribosomal RNA gene, partial sequence (FJ898313)	spring	89%	Bosea thiooxidans (EU730912)	88%
T002	D	FR796656	Bacteriodetes	Uncultured bacterium clone G910P35FB10.T0 16S ribosomal RNA gene, partial sequence (EU172242)	air	84%	Sediminibacterium ginsengisoli (EF067860)	84%
T035	D	FR796689	Bacteriodetes	Uncultured bacterium clone TLM10/TLMdgge01 16S ribosomal RNA gene, partial sequence (AF534434)	Toolik Lake main station at 3 m depth	97%	Adhaeribacter aquaticus (AJ626894)	85%
T036	D	FR796690	Bacteriodetes	Uncultured bacterium isolate SSCP band GR1-RNA1-3-27 16S ribosomal RNA, partial sequence (DQ077593)	drinking water distribution system HWW	99%	Sejongia jeonii (AY553294)	83%
T037	R	FR796691	Bacteriodetes	Uncultured Bacteroidetes bacterium partial 16S rRNA gene, clone JG35-K2-AG43 (AM403313)	soil	93%	Flexibacter canadensis (AB078046)	85%
T038	D	FR796692	Bacteriodetes	Uncultured Bacteroidetes bacterium clone IRD18D04 16S ribosomal RNA gene, partial sequence (AY947930)	USA: Massachusetts, Ipswich River	95%	Ekhidna lutea (AM746475)	86%
T039	D	FR796693	Bacteriodetes	Uncultured bacterium isolate SSCP band TW16-D_14_9 16S ribosomal RNA gene, partial sequence (DQ077625)	drinking water distribution system HWW	88%	Sediminibacterium salmoneum (EF407879)	87%
T040	D	FR796694	Bacteriodetes	Uncultured Bacteroidetes bacterium partial 16S rRNA gene, clone NE02 (AJ575726)	Grosse Fuchskuhle	90%	Sediminibacterium ginsengisoli (EF067860)	90%

T041	D	FR796695	Bacteriodetes	Uncultured Sphingobacteria bacterium clone LW9m-1-10 16S ribosomal RNA gene, partial sequence (EU641456)	Lake Michigan	98%	Terrimonas ferruginea (AM230484)	93%
T042	D	FR796696	Bacteriodetes	Uncultured bacterium isolate SSCP band TW16-D_14_9 16S ribosomal RNA gene, partial sequence (DQ077625)	drinking water distribution system HWW	93%	Sediminibacterium salmoneum (EF407879)	92%
T043	D	FR796697	Bacteriodetes	Uncultured bacterium isolate SSCP band TW16-D_14_9 16S ribosomal RNA gene, partial sequence (DQ077625)	drinking water distribution system HWW	100%	Sediminibacterium salmoneum (EF407879)	97%
T044	D	FR796698	Bacteriodetes	Uncultured bacterium isolate SSCP band TW16-D_14_9 16S ribosomal RNA gene, partial sequence (DQ077625)	drinking water distribution system HWW	98%	Sediminibacterium salmoneum (EF407879)	96%
T001	R	FR796655	Betaproteobacteria	Uncultured bacterium isolate SSCP band GT-8R_03-20 16S ribosomal RNA, partial sequence (DQ077557)	drinking water distribution system HWW	97%	Candidatus Tremblaya princeps (AF476079)	97%
T007	D	FR796661	Betaproteobacteria	Uncultured beta proteobacterium partial 16S rRNA gene, isolate DGGE band WETLE-13B (FM991990)	constructed wetland	87%	Candidatus Accumulibacter phosphatis (AY962316)	87%
T008	D	FR796662	Betaproteobacteria	Uncultured bacterium clone DP10.5.4 16S ribosomal RNA gene, partial sequence (FJ612426)	lake water	100%	Methylophilus methylotrophus (GQ175365)	96%
T009	R	FR796663	Betaproteobacteria	Nitrosospira briensis 16S ribosomal RNA gene, partial sequence (AY123800)	rhizosphere	100%	Nitrosospira briensis (AY123800)	100%
T010	R	FR796664	Betaproteobacteria	Uncultured bacterium partial 16S rRNA gene, clone SZB2 (AM176880)	mangrove sediment	98%	Oxalicibacterium flavum (AY061962)	94%
T011	D	FR796665	Betaproteobacteria	Uncultured beta proteobacterium clone PRD18F04 16S ribosomal RNA gene, partial sequence (AY948047)	USA: Massachusetts, Parker River	99%	Acidovorax facilis (EU730927)	99%
T012	R	FR796666	Betaproteobacteria	Uncultured bacterium isolate SSCP band RNA2-9-10 16S ribosomal RNA, partial sequence (DQ077559)	drinking water distribution system HWW	96%	Acidovorax facilis (GQ284412)	95%

T013	D	FR796667	Betaproteobacteria	Uncultured beta proteobacterium clone CB31D01 16S ribosomal RNA gene, partial sequence (EF471693)	whole surface water from Chesapeake bay	97%	Kerstersia gyiorum (AY131213)	97%
T014	D	FR796668	Betaproteobacteria	Uncultured Polynucleobacter sp. isolate SSCP band 155-0-5 16S ribosomal RNA gene, partial sequence (GU088519)	water sample from Sumauma river	86%	Polynucleobacter necessarius subsp. asymbioticus (CP001010)	86%
T029	R	FR796683	Cyanobacteria	Uncultured bacterium isolate SSCP band TW15-RNA1-14-2 16S ribosomal RNA, partial sequence (DQ077556)	drinking water distribution system HWW	100%	Gracilibacter thermotolerans (DQ117469)	85%
T030	R	FR796684	Cyanobacteria	Uncultured bacterium isolate SSCP band PT_27 16S ribosomal RNA gene, partial sequence (GQ917147)	drinking water distribution system HWW	93%	Megamonas rupellensis (EU346729)	81%
T031	R	FR796685	Cyanobacteria	Uncultured bacterium isolate SSCP band PT_27 16S ribosomal RNA gene, partial sequence (GQ917147)	drinking water distribution system HWW	87%	Methylobacter tundripaludum (AJ414655)	85%
T032	R	FR796686	Gammaproteobacteria	Methylobacter tundripaludum 16S ribosomal RNA, type strain SV96T (AJ414655)	iron-rich snow	99%	Methylobacter tundripaludum (AJ414655)	99%
T033	R	FR796687	Gammaproteobacteria	Uncultured bacterium isolate SSCP band RNA2-8-7 16S ribosomal RNA, partial sequence (DQ077602)	drinking water distribution system HWW	99%	Methylocaldum gracile (U89298)	92%
T022	R	FR796676	Nitrospira	Uncultured Nitrospira sp. clone I-GAC-2 16S ribosomal RNA gene, partial sequence (GQ452974)	drinking water	93%	Nitrospira moscoviensis (X82558)	91%
T005	D	FR796659	Planctomycetes	Uncultured bacterium gene for 16S rRNA, partial sequence, clone: MIZ10 (AB179501)		89%	Mycoplasma dispar (AF412979)	83%
T006	D	FR796660	Planctomycetes	Uncultured bacterium isolate SSCP band GT-8R_03-21 16S ribosomal RNA, partial sequence (DQ077597)	drinking water distribution system HWW	85%	Nevskia ramosa (AJ001011)	88%
T034	R	FR796688	Planctomycetes	Uncultured bacterium clone N1-103 16S ribosomal RNA gene, partial sequence (EU443041)	Nam Co Lake water	99%	Gemmata obscuriglobus (X85248)	87%

T023	R	FR796677	TM6	Uncultured bacterium gene for 16S ribosomal RNA, partial sequence, isolate: DGGE band: 8 (AB472269)	crude oil-contaminated soil	82%	Candidatus Glomeribacter gigasporarum (AM889131)	87%
T024	R	FR796678	TM6	Uncultured bacterium isolate SSCP band PT_19 16S ribosomal RNA gene, partial sequence (GQ917139)	drinking water distribution system HWW	94%	Desulfococcus biacutus (AJ277887)	88%
T025	R	FR796679	TM6	Uncultured bacterium clone SGSH795 16S ribosomal RNA gene, partial sequence (GQ347607)	Saanich Inlet, 215 m depth	93%	Nevskia ramosa (AJ001343)	87%

Supplementary Table 2: Taxonomic identification of single phylotypes found in biofilm SSCP fingerprints shown in Figure 4.4a. Sample origin: D, only present in DNA-based fingerprints; R, only present in RNA-based fingerprints; RD, present in DNA and RNA-based fingerprints.

Phylotype designation	Sample origin	GenBank accession no.	Taxonomic group	Closest 16S rRNA gene sequence (Accession no.)	source of closest sequence	% Similarity	Closest described species (Accession no.)	% Similarity
B016	R	FR796558	Acidobacteria	Uncultured bacterium gene for 16S rRNA, partial sequence, clone: 1333 (AB286500)	activated sludge	99%	Candidatus Solibacter usitatus Ellin6076 (CP000473)	93%
B017	R	FR796559	Acidobacteria	Uncultured bacterium 16S rRNA gene, clone D14305 (AJ617855)	oxic-anoxic interphase of flooded paddy soil	99%	Candidatus Solibacter usitatus (GQ287529)	91%
B048	D	FR796590	Acidobacteria	Uncultured bacterium clone FFCH3013 16S ribosomal RNA gene, partial sequence (EU132279)	soil from an undisturbed mixed grass prairie reserve	86%	Eubacterium siraeum (EU266550)	87%
B049	R	FR796591	Acidobacteria	Uncultured bacterium clone FFCH3013 16S ribosomal RNA gene, partial sequence (EU132280)	soil from an undisturbed mixed grass prairie reserve	91%	Tepidanaerobacter syntrophicus (AB106354)	88%
B050	R	FR796592	Acidobacteria	Uncultured bacterium clone 4 16S ribosomal RNA gene, partial sequence (EU362133)	dune sand	88%	Candidatus Desulforudis audaxviator MP104C (CP000860)	85%
B052	R	FR796594	Acidobacteria	Uncultured bacterium clone AK1DE1_02D 16S ribosomal RNA gene, partial sequence (GQ396963)	soil	77%	Agrobacterium tumefaciens (AF508099)	76%
B054	D	FR796596	Acidobacteria	Uncultured bacterium clone AK1AB1_12H 16S ribosomal RNA gene, partial sequence (GQ396847)	soil	97%	Selenomonas sputigena (GQ422723)	91%

B057	D	FR796599	Acidobacteria	Uncultured bacterium clone 099.F24 16S ribosomal RNA gene, partial sequence (EU355855)	agricultural soil treatment	92%	Desulfovibrio giganteus (AF418170)	88%
B058	D	FR796600	Acidobacteria	Uncultured delta proteobacterium clone MPWIC_G06 16S ribosomal RNA gene, partial sequence (EF414165)	sponge	89%	Desulfovibrio indonesiensis (Y09504)	85%
B033	D	FR796575	Actinobacteria	Uncultured bacterium clone LL141-8C18 16S ribosomal RNA gene, partial sequence (FJ675406)	feedlot surface material	98%	Thermoleophilum album (AJ458462)	88%
B083	D	FR796625	Actinobacteria	Uncultured bacterium clone TX5A_90 16S ribosomal RNA gene (FJ152798)	alkaline saline soils of the former lake Texcoco	100%	Iamia majanohamensis (AB360448)	92%
B084	D	FR796626	Actinobacteria	Uncultured bacterium clone TX5A_90 16S ribosomal RNA gene (FJ152798)	alkaline saline soils of the former lake Texcoco	90%	Iamia majanohamensis (AB360448)	83%
B085	D	FR796627	Actinobacteria	Uncultured bacterium clone LL141-8C18 16S ribosomal RNA gene (FJ675406)	feedlot surface material	98%	Thermoleophilum album (AJ458462)	88%
B010	R	FR796552	Alphaproteobacteria	Uncultured alpha proteobacterium clone GASP-MA4W2_F12 16S ribosomal RNA gene, partial sequence (EF664356)	grassland	78%	Skermanella aerolata strain 5416T-32 (DQ672568)	72%
B011	R	FR796553	Alphaproteobacteria	Uncultured soil bacterium clone Bact.dry.ACETF05 16S ribosomal RNA gene, partial sequence (GU375766)	oil-field soil	94%	Candidatus Reyranella massiliensis (EF394922)	89%
B013	D	FR796555	Alphaproteobacteria	Wolbachia endosymbiont of Cubitermes sp. clone T5 16S ribosomal RNA gene, partial sequence (EF417899)	Cubitermes sp	78%	Wolbachia pipientis strain wHa (DQ235279)	77%
B021	R	FR796563	Alphaproteobacteria	Uncultured bacterium clone F126 16S ribosomal RNA gene, partial sequence (FJ348594)	activated sludge	98%	Clostridium sporogenes (L09175)	91%

B059	D	FR796601	Alphaproteobacteria	Uncultured Alphaproteobacteria bacterium 16S rRNA gene from clone QEDQ2AE01 (CU923390)	municipal wastewater sludge	64%	Serratia marcescens (FM213393)	88%
B061	D	FR796603	Alphaproteobacteria	Uncultured bacterium gene for 16S rRNA, partial sequence, clone:14s (AB154445)	PCR-derived sequence from meromictic lake sediment	93%	Desulfomonile limimaris (AF282177)	91%
B062	D	FR796604	Alphaproteobacteria	Uncultured alpha proteobacterium partial 16S ribosomal RNA, clone SSCP ribotype S-RS-13b (AM989610)	soddy-podzolic soil casts of earthworms	89%	Mesorhizobium thiogangeticum (AJ864462)	86%
B063	D	FR796605	Alphaproteobacteria	Uncultured alpha proteobacterium partial 16S ribosomal RNA, clone SSCP ribotype S-RS-13b (AM989610)	soddy-podzolic soil casts of earthworms	87%	Pseudoxanthobacter soli (EF465533)	85%
B064	D	FR796606	Alphaproteobacteria	Hyphomicrobium sp. D3 16S ribosomal RNA gene, partial sequence (EF079086)	ditch sediment collected in freshwater	88%	Azospirillum brasilense (Z29617)	90%
B065	R	FR796607	Alphaproteobacteria	Bacterium Fuku2-ISO-153 16S ribosomal RNA gene, partial sequence (EU409478)	Lake Fuchskuhle	92%	Hyphomicrobium facile (Y14312)	88%
B066	RD	FR796608	Alphaproteobacteria	Uncultured bacterium clone 1C227168 16S ribosomal RNA gene, partial sequence (EU799556)	Newport Harbour, RI	99%	Rhodobacter blasticus (DQ342322)	97%
B067	R	FR796609	Alphaproteobacteria	Uncultured bacterium clone 1C227168 16S ribosomal RNA gene, partial sequence (EU799556)	Newport Harbour, RI	99%	Rhodobacter capsulatus (DQ342320)	98%
B068	R	FR796610	Alphaproteobacteria	Uncultured bacterium clone nbw1075f08c1 16S ribosomal RNA gene, partial sequence (GQ052877)	skin, volar forearm	85%	Rhodobacter blasticus (DQ342322)	84%
B069	R	FR796611	Alphaproteobacteria	Uncultured bacterium clone H07_SE4A 16S ribosomal RNA gene, partial sequence (FJ592547)	wetland soil	93%	Rhodovulum marinum (AM696693)	91%
B070	R	FR796612	Alphaproteobacteria	Uncultured alpha proteobacterium partial 16S rRNA gene, clone h5-3 (FN594682)	biofilm from gold mine in Zloty Stok	89%	Woodsholea maritima (AJ578477)	86%

B071	RD	FR796613	Alphaproteobacteria	Uncultured bacterium gene for 16S rRNA, partial sequence, clone: SWB05 (AB294316)	stream	100%	Novosphingobium stygium (U20775)	94%
B072	D	FR796614	Alphaproteobacteria	Uncultured bacterium partial 16S rRNA gene, isolate DGGE_band_ag8 (AM749502)	lysimeter soil	88%	Anderseniella baltica (AM712634)	89%
B073	D	FR796615	Alphaproteobacteria	Uncultured alpha proteobacterium partial 16S rRNA gene, clone d5-3 (FN594655)	biofilm from gold mine in Zloty Stok	97%	Pedomicrobium australicum (FM886896)	94%
B074	RD	FR796616	Alphaproteobacteria	Uncultured bacterium clone WW1_b1 16S ribosomal RNA gene, partial sequence (GQ264217)	simulated low level waste site	94%	Hyphomicrobium sulfonivorans (AY468372)	97%
B075	D	FR796617	Alphaproteobacteria	Uncultured bacterium clone WW1_b1 16S ribosomal RNA gene (GQ264217)	simulated low level waste site	94%	Hyphomicrobium sulfonivorans (AY468372)	93%
B076	R	FR796618	Alphaproteobacteria	Uncultured alpha proteobacterium partial 16S rRNA gene, clone e10-1 (FN594694)	biofilm from gold mine in Zloty Stok	92%	Hyphomicrobium zavarzinii (Y14306)	91%
B077	R	FR796619	Alphaproteobacteria	Uncultured alpha proteobacterium clone as2-53 16S ribosomal RNA (GU257609)	activated sludge in a membrane bioreactor	92%	Hyphomicrobium sulfonivoran (AY468372)	90%
B078	D	FR796620	Alphaproteobacteria	Uncultured bacterium clone Bul1ah07 16S ribosomal RNA gene (FJ228809)	host:Bulinus africanus	96%	Bradyrhizobium yuanmingense (FJ785218)	92%
B079	D	FR796621	Alphaproteobacteria	Uncultured alpha proteobacterium clone A23YM01RM small subunit (FJ569518)	soil early snow melt site B, Alpes	92%	Rhodoplanes piscinae (AM712913)	90%
B080	D	FR796622	Alphaproteobacteria	Uncultured bacterium clone TY-R-II-OTU6 16S ribosomal RNA gene (FJ178175)	soil derived from quaternary red clay	93%	Rhodoplanes piscinae (AM712913)	91%
B081	R	FR796623	Alphaproteobacteria	Uncultured bacterium clone MACA-RR12 16S ribosomal RNA gene (GQ500747)	base level cave stream, Roaring River (sediment)	84%	Rhodobium orientis (D30792)	83%

B082	RD	FR796624	Alphaproteobacteria	Uncultured bacterium gene for 16S rRNA, partial sequence, clone: 136 (AB286364)	activated sludge	98%	Hyphomicrobium sulfonivorans (AY305006)	88%
B109	R	FR796651	Alphaproteobacteria	Uncultured alpha proteobacterium clone J-DW-21 16S ribosomal RNA gene, partial sequence (GQ453317)	Surface Water Treatment Plant	96%	Parvularcula lutaonensis (EU346850)	90%
B089	D	FR796631	Bacteriodetes	Uncultured Flexibacteraceae bacterium clone BL017B17 16S ribosomal (DQ188271)	bronchoalveolar lavage fluid from children with cystic fibrosis	93%	Pontibacter korlensis (GQ503321)	86%
B090	RD	FR796632	Bacteriodetes	Uncultured Bacteroidetes bacterium clone I-GAC-12 16S ribosomal RNA (GQ452967)	drinking water treatment plant	99%	Terrimonas ferruginea (AM230484)	98%
B091	RD	FR796633	Bacteriodetes	Uncultured Bacteroidetes bacterium clone I-GAC-13 16S ribosomal RNA (GQ452968)	drinking water treatment plant	99%	Terrimonas ferruginea (AM230484)	93%
B098	D	FR796640	Betaproteobacteria	Uncultured bacterium clone GB062005_2-35 16S ribosomal RNA gene (GQ487819)	groundwater surface water interface sediments	93%	Azospira restricta (DQ974114)	90%
B099	D	FR796641	Betaproteobacteria	Sterolibacterium sp. TKU1 partial 16S rRNA gene, strain TKU1 (AM990454)	ultra pure water from an industrial cooling water system	89%	Sideroxydans lithotrophicus (DQ386859)	85%
B100	R	FR796642	Betaproteobacteria	Uncultured Burkholderiales bacterium clone Gap-2-58 16S ribosomal RN (EU642196)	Lake Michigan	100%	Methylibium aquaticum (DQ664244)	99%
B101	RD	FR796643	Betaproteobacteria	Uncultured Burkholderiales bacterium partial 16S rRNA gene, clone B6_93 (AM940846)	glacier moraine	100%	Methylibium fulvum (AB245356)	100%
B102	D	FR796644	Betaproteobacteria	Uncultured bacterium clone AK4DE1_01F 16S ribosomal RNA gene (GQ397030)	soil	98%	Thiobacillus thiophilus (EU685841)	92%

B103	R	FR796645	Betaproteobacteria	Uncultured beta proteobacterium clone D10_37 small subunit ribosomal (EU266802)	tar-oil contaminated aquifer sediments	97%	Burkholderia andropogonis (DQ786950)	95%
B104	RD	FR796646	Betaproteobacteria	Uncultured bacterium clone MYS8 16S ribosomal RNA gene (GU305733)	MiYu reservoir water (oligotrophic lake)	98%	Ultramicrobacter hongkongensis (DQ532120)	97%
B105	D	FR796647	Betaproteobacteria	Uncultured Antarctic bacterium LB3-81 16S ribosomal RNA gene, partial sequence (AF173823)	Permanent Antarctic Lake Ice	97%	Derxia gummosa (AB089482)	94%
B106	R	FR796648	Betaproteobacteria	Uncultured bacterium clone nbw877d04c1 16S ribosomal RNA gene, partial sequence (GQ030383)	skin, elbow	93%	Ralstonia syzygii (AY464966)	93%
B107	R	FR796649	Betaproteobacteria	Uncultured beta proteobacterium clone GASP-WB2S1_E03 16S ribosomal RNA gene, partial sequence (EF073875)	pasture	78%	Nitrosospira multiformis ATCC 25196 (CP000103)	75%
B001	R	FR796543	Chlamydiales	Uncultured Chlamydiales bacterium clone P-7 16S ribosomal RNA gene, partial sequence (AF364569)	environmental sample	100%	Neochlamydia hartmannellae strain A1Hsp (NR_025037)	90%
B002	R	FR796544	Chlamydiales	Uncultured soil bacterium clone 530-2 small subunit ribosomal RNA gene, partial sequence (AY326519)	soil	93%	Parachlamydia acanthamoebae strain CRIB43 (FJ532291)	91%
B003	R	FR796545	Chlamydiales	Uncultured bacterium clone MABRDTU43 16S ribosomal RNA gene, partial sequence (FJ529996)	autotrophic nitrifying biofilm reactor	99%	Parachlamydia acanthamoebae strain CRIB44 (FJ532291.2)	91%
B004	R	FR796546	Chlamydiales	Parachlamydiaceae bacterium CHSL 16S ribosomal RNA gene, partial sequence (GQ221847)	hartmannellid amoeba SL-2	93%	Parachlamydia acanthamoebae strain CRIB45 (FJ532291.3)	92%
B005	R	FR796547	Chlamydiales	Candidatus Protochlamydia sp. CRIB40 16S ribosomal RNA gene, partial sequence (FJ532293)	biofilm from clarifier	98%	Protochlamydia naegleriophila strain CRIB42 (FJ532295)	96%

B006	R	FR796548	Chlamydiales	Uncultured bacterium gene for 16S rRNA, partial sequence, clone: NG_inoculum_16 (AB518086)	activated sludge	93%	Neochlamydia hartmannellae strain A1Hsp (NR_025037)	92%
B007	D	FR796549	Chlamydiales	Uncultured Chlamydiae bacterium clone DSM2W1u70 16S ribosomal RNA gene, partial sequence (EU635381)	showerhead swab	84%	Candidatus Fritschea bemisiae strain Falk (AY140910)	81%
B008	RD	FR796550	Chlamydiales	Endosymbiont of Acanthamoeba sp. R18 gene for 16S rRNA, partial sequence (AB506679)	Acanthamoeba sp. R18	90%	Protochlamydia naegleriophila strain CRIB41 (FJ532294)	90%
B009	D	FR796551	Chlamydiales	Candidatus Protochlamydia sp. cvE12 16S ribosomal RNA gene, partial sequence (FJ976092)	fountain	86%	Parachlamydia acanthamoebae strain CRIB43 (FJ532291)	88%
B020	R	FR796562	Chlamydiales	Uncultured bacterium gene for 16S rRNA, partial sequence, clone: PltcGammaproteobacterium88 (AB424911)	hydrothermal sulfide structure	88%	Bacillus megaterium (EU910239)	86%
B024	D	FR796566	Chloroflexi	Uncultured Chloroflexi bacterium clone AKYG631 16S ribosomal RNA gene, partial sequence (AY921657)	farm soil adjacent to a silage storage bunker	93%	Thermanaeromonas toyohensis (AB062280)	87%
B025	D	FR796567	Chloroflexi	Uncultured bacterium clone 344.F22 16S ribosomal RNA gene, partial sequence (EU353968)	agricultural soil treatment	100%	Thermaerobacter composti (AB454087)	86%
B026	D	FR796568	Chloroflexi	Uncultured bacterium clone 344.F22 16S ribosomal RNA gene, partial sequence (EU353968)	agricultural soil treatment	91%	Ureibacillus thermophilus (DQ348072)	86%
B108	R	FR796650	Chloroflexi	Uncultured Chloroflexi bacterium partial 16S rRNA gene, clone AMJA2 (AM934855)	hydrocarbon-contaminated soil	91%	Moorella perchloratireducens (EF060194)	91%
B056	D	FR796598	Deltaproteobacteria	Uncultured bacterium clone C9 G3 16S ribosomal RNA gene, partial sequence (GU366869)	temperate forest soil	87%	Geobacter sulfurreducens (U13928)	84%

B060	R	FR796602	Deltaproteobacteria	Uncultured delta proteobacterium clone TDNP_USbc97_138_1_18 16S ribosomal RNA gene, partial sequence (FJ516890)	upper sediment	88%	Rhodospirillum sulfurexigens (AM710622)	88%
B012	R	FR796554	Firmicutes	Uncultured bacterium isolate SSCP band PT_27 16S ribosomal RNA gene, partial sequence (GQ917147)	HWW drinking water	95%	Leptolyngbya frigida (AY493574)	87%
B022	D	FR796564	Firmicutes	Uncultured bacterium clone 1-gw2-su4-12 16S ribosomal RNA gene, partial sequence (DQ981803)	river water	79%	Bacillus licheniformis (X68416)	84%
B023	D	FR796565	Firmicutes	Bacillus sp. PLC9 16S ribosomal RNA gene, partial sequence (FJ973430)	treatment water	91%	Bacillus pichinotyi (EU373388)	90%
B051	R	FR796593	Firmicutes	Uncultured Clostridiaceae bacterium gene for 16S rRNA, clone: dgD-50 (AB264067)	PCR-derived from Dugong feces	89%	Anaerosporobacter mobilis (AY534872)	88%
B053	D	FR796595	Firmicutes	Uncultured bacterium clone 16_14D09 16S ribosomal RNA gene, partial sequence (GQ360313)	left upper lung lobe	87%	Ethanoligenens harbinense (EU639425)	86%
B014	RD	FR796556	Gammaproteobacteria	Uncultured bacterium clone WC3_79 16S ribosomal RNA gene, partial sequence (GQ264139)	simulated low level waste site	91%	Legionella rubrilucens (Z32643)	90%
B015	D	FR796557	Gammaproteobacteria	Uncultured Legionellaceae bacterium isolate DGGE gel band M4-3(I) 16S ribosomal RNA gene, partial sequence (FJ467409)	bovine mastitis milk	95%	Legionella lytica (Z49741)	94%
B034	D	FR796576	Gammaproteobacteria	Uncultured bacterium clone A1-07 16S ribosomal RNA gene, partial sequence (EU857839)	Ross Sea sediment	82%	Propionivibrio limicola (AJ307983)	83%
B035	R	FR796577	Gammaproteobacteria	Uncultured gamma proteobacterium clone NE41C01cA 16S ribosomal RNA gene, partial sequence (DQ424446)	microbial mat	85%	Sedimenticola selenatireducens (AF432145)	84%
B055	D	FR796597	Gammaproteobacteria	Uncultured gamma proteobacterium clone NE36D07cA 16S ribosomal RNA gene, partial sequence (DQ424244)	microbial mat	85%	Hyphomicrobium zavarzinii (Y14306)	85%

B086	D	FR796628	Gammaproteobacteria	Uncultured bacterium clone C2 A14 16S ribosomal RNA gene (GU366816)	temperate forest soil	88%	Thioalkalivibrio denitrificans (AF126545)	89%
B092	D	FR796634	Gammaproteobacteria	Uncultured bacterium clone D44 16S ribosomal RNA gene (EU234314)	upstream of Wang Yang River	83%	Panacagrimonas perspica (AB257720)	79%
B093	D	FR796635	Gammaproteobacteria	Uncultured bacterium clone D44 16S ribosomal RNA gene (EU234314)	upstream of Wang Yang River	91%	Hydrocarboniphaga effusa (AY363244)	88%
B094	R	FR796636	Gammaproteobacteria	Uncultured bacterium clone D44 16S ribosomal RNA gene (EU234314)	upstream of Wang Yang River	98%	Panacagrimonas perspica (AB257720)	90%
B095	R	FR796637	Gammaproteobacteria	Uncultured sulfur-oxidizing symbiont bacterium partial 16S rRNA gene (AM935643)	hydrocarbon-contaminated soil	94%	Halochromatium glycolicum (X93472)	89%
B096	RD	FR796638	Gammaproteobacteria	Uncultured bacterium clone dr61 16S ribosomal RNA gene (AY540779)	gold mine south africa	97%	Thiohalomonas denitrificans (EF117913)	93%
B097	D	FR796639	Gammaproteobacteria	Uncultured gamma proteobacterium clone A19YC01RM small subunit (FJ568372)	soil early snow melt site B, Alpes	96%	Steroidobacter denitrificans (EF605262)	88%
B018	D	FR796560	Nitrospira	Uncultured bacterium isolate SSCP band TW16-8R-16-6 16S ribosomal RNA, partial sequence (DQ077576)	drinking water distribution system HWW	100%	Nitrospira moscoviensis (X82558)	97%
B019	D	FR796561	Nitrospira	Uncultured bacterium DSSD16 16S ribosomal RNA gene, partial sequence (AY328715)	drinking water distribution system simulator	100%	Nitrospira moscoviensis (X82559)	99%
B027	D	FR796569	Planctomycetes	Uncultured planctomycete partial 16S rRNA gene, isolate OTU32/APA (AM902610)	subsurface thermal spring	97%	Zavarzinella formosa (AM162406)	88%
B028	D	FR796570	Planctomycetes	Uncultured bacterium clone F2_116X 16S ribosomal RNA gene, partial sequence (GQ262993)	simulated low level waste site	87%	Gemmata obscuriglobus (X56305)	90%

B029	D	FR796571	Planctomycetes	Uncultured bacterium clone FFCH1421 16S ribosomal RNA gene, partial sequence (EU135075)	soil from an undisturbed mixed grass prairie reserve	99%	Thermodesulfovibrio hydrogeniphilus (EF081294)	87%
B030	D	FR796572	Planctomycetes	Uncultured planctomycete clone PL09-10 16S ribosomal RNA gene, partial sequence (FJ844345)	water of high-mountain lake	97%	Pirellula staleyi (X81948)	87%
B031	D	FR796573	Planctomycetes	Uncultured bacterium NewOrleansYard3_YD3_032406_139 16S ribosomal RNA gene, partial sequence (FJ525164)	sediment	85%	Pirellula staleyi (X81948)	78%
B032	R	FR796574	Planctomycetes	Uncultured bacterium clone FW026-181 16S ribosomal RNA gene, partial sequence (EF692781)	sediment	91%	Subaequorebacter tamlense (AM293856)	85%
B087	R	FR796629	Planctomycetes	uncultured bacterium gene for 16S rRNA, partial sequence, clone:OS-54 (AB205985)	activated sludge	87%	Escherichia coli strain BEE25 16S ribosomal RNA gene (EF560792)	83%
B088	D	FR796630	Planctomycetes	Uncultured bacterium clone 168b1 16S ribosomal RNA gene (EF459840)	Baltic Sea sediment	83%	Nevskia soli (EF178286)	86%
B036	D	FR796578	TM6	Uncultured bacterium partial 16S rRNA gene, clone HA5-SRB-c056 (FM868200)	sediment	85%	Thiodictyon elegans (EF999973)	86%
B037	R	FR796579	TM6	Uncultured bacterium clone MD2902-B36 16S ribosomal RNA gene, partial sequence (EU385862)	subseafloor sediment of the South China sea	91%	Panacagrimonas perspica (AB257720)	81%
B038	R	FR796580	TM6	Uncultured division TM6 bacterium clone NOS7.2WL 16S ribosomal RNA gene, partial sequence (AY043739)	forest cut-block surface organic matter	94%	Eubacterium rangiferina (EU124830)	82%
B039	RD	FR796581	TM6	Uncultured bacterium clone FGL7S_B80 16S ribosomal RNA gene, partial sequence (FJ437950)	Green Lake surface sediments	90%	Syntrophothermus lipocalidus (AB021305)	88%
B040	R	FR796582	TM6	Uncultured soil bacterium clone 331 16S ribosomal RNA gene, partial sequence (EU106159)	soil from radish rich area	91%	Rubrobacter radiotolerans (AJ243870)	88%

B041	R	FR796583	TM6	Uncultured bacterium partial 16S rRNA gene, clone a7-4 (FN594638)	biofilm from gold mine in Zloty Stok	93%	Desulfococcus multivorans (AF418173)	87%
B042	RD	FR796584	TM6	Uncultured candidate division TM6 bacterium partial 16S rRNA gene, clone CM1F08 (AM936568)	hydrocarbon-contaminated soil	92%	Eubacterium yurii subsp. schtitka (AY533382)	88%
B043	R	FR796585	TM6	Uncultured bacterium clone I-GAC-3 16S ribosomal RNA gene, partial sequence (GQ452985)	water from drinking water treatment plant	88%	Hydrocarboniphaga effusa (AY363245)	90%
B044	D	FR796586	TM6	Uncultured bacterium clone PP4-50 16S ribosomal RNA gene, partial sequence (EU148985)	prepupa gut	94%	Wohlfahrtiimonas chitiniclastica (EU484335)	87%
B045	D	FR796587	TM6	Uncultured soil bacterium clone CWT SM03_G11 16S ribosomal RNA gene, partial sequence (DQ129127)	Coweeta forest soil	91%	Wohlfahrtiimonas chitiniclastica (EU484335)	86%
B046	R	FR796588	TM6	Uncultured candidate division TM6 bacterium clone DSR2W1u09 16S ribosomal RNA gene, partial sequence (EU635154)	showerhead swab	96%	Rubrobacter radiotolerans (AJ243870)	87%
B047	R	FR796589	TM6	Uncultured soil bacterium clone CWT SM03_G11 16S ribosomal RNA gene, partial sequence (DQ129127)	Coweeta forest soil	97%	Wohlfahrtiimonas chitiniclastica (EU484335)	86%

Supplementary Tab. 3: Number of retrieved phylotypes from bulk water and biofilms (presence/absence data). DNA: Number of phylotypes found in DNA-based fingerprints. RNA: Number of phylotypes found in RNA-based fingerprints. DNA+RNA: Number of phylotypes found in both types of fingerprints.

Phylum / Class	**Bulk water**				**Biofilm**			
	DNA	**RNA**	**DNA+RNA**	**Total**	**DNA**	**RNA**	**DNA+RNA**	**Total**
Acidobacteria	7	0	0	7	4	5	0	9
Actinobacteria	0	0	0	0	6	0	0	6
Bacteriodetes	10	1	0	11	3	2	2	3
Candidate division TM6	0	3	0	3	5	9	2	12
Chlamydiales	0	0	0	0	3	8	1	10
Chloroflexi	0	0	0	0	4	1	0	5
Cyanobacteria	0	3	0	3	0	0	0	0
Firmicutes	0	0	0	0	3	2	0	5
Nitrospira	0	1	0	1	2	0	0	2
Planctomycetes	2	1	0	3	6	2	0	8
Proteobacteria:								
Alphaproteobacteria	2	3	0	5	16	16	4	28
Betaproteobacteria	5	4	0	9	6	6	2	10
Gammaproteobacteria	0	2	0	2	9	5	2	12
Deltaproteobacteria	0	0	0	0	1	1	0	2
Total	26	18	0	44	68	57	13	112

Chapter 5

5 Assessing the viability of bacterial species in drinking water by combined cellular and molecular analyses

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5.1 Abstract

The question which bacterial species are present in drinking water and if they are viable is essential for drinking water safety. To approach this question we combined Propidium iodide/SYTO9 staining ("live/dead staining" indicating membrane integrity), Fluorescence Activated Cell Sorting (FACS) and community fingerprinting for the analysis of a set of tap water samples. Live/dead staining revealed that about half of the bacteria in the tap water had intact membranes. Molecular analysis using 16S rRNA and 16S rRNA gene-based single strand conformation polymorphism (SSCP) fingerprints and sequencing of drinking water bacteria before and after FACS sorting revealed: i) the DNA- and RNA-based overall community structure differed substantially, ii) the community retrieved from RNA and DNA reflected different bacterial species, classified as 53 phylotypes (with only two common phylotypes), iii) the percentage of phylotpes with intact membranes or damaged cells were comparable for RNA and DNA based analyses, and iv) the retrieved species were primarily of aquatic origin. The pronounced difference between community structure phylotypes obtained from DNA extracts (dominated by *Betaproteobacteria, Bacteroidetes* and *Actinobacteria*) and from RNA extracts (dominated by *Alpha-, Beta-, Gammaproteobacteria, Bacteroidetes* and *Cyanobacteria*) demonstrate the relevance of concomitant RNA and DNA analyses for drinking water studies. Unexpected was that a comparable fraction (about 21%) of phylotypes with membrane injured cells was observed for DNA- and RNA-based analyses, contradicting the current understanding that RNA-based analyses represent the actively growing fraction of the bacterial community. Overall, we think that this combination of "live" staining, FACS sorting and molecular analysis opens new avenues for functional fingerprinting.

5.2 Introduction

Drinking water commonly provides a diverse microflora to the end user despite the fact that water processing eliminates a large fraction of microorganisms present in raw water, as shown by detailed molecular studies (14, 32). Bacteria originating from source water, regrowth in bulk water and biofilms of the distribution network contribute to the generation of a diverse bacterial community in drinking water (17).

Molecular methods, such as 16S rRNA-based and 16S rRNA gene-based fingerprints, can provide an overview on the bacterial community and thus can overcome the restriction of cultivation based methods that detect only the few bacteria growing under the respective cultivation conditions (7). These molecular methods allow overcoming the problem of non-culturability for viable-but-non-culturable (VBNC) bacteria, i.e. even under adequate cultivation conditions these bacteria do not grow due to physiological constraints (21). However, molecular methods based on extracted nucleic acids cannot distinguish between live and dead bacteria (5, 28). During the last years, a broad set of fluorescent stains was developed allowing insight into the physiological state of bacteria (22). Stains assessing membrane integrity, such as Propidium Iodide (PI) and SYTO9, are considered to distinguish between membrane intact and membrane injured cells (6). This staining procedure has been evaluated and compared by a set of studies to other staining procedures for assessment of the physiological state of the bacteria (3, 11, 22). Membrane injury was evaluated as a reliable criterion for cell death where recovery is highly unlikely.

Bacterial community fingerprints and subsequent sequencing of the single fingerprint bands followed by phylogenetic analysis provide an overview on the structure and composition of bacterial drinking water communities (14). Single bacterial species can be detected by these fingerprints, e.g. from 16S rRNA-based Single Strand Conformational Polymorphism (SSCP) analysis, at a relative abundance of 0.1% and more using general bacterial 16S rRNA gene primers. Besides providing an overview, fingerprints allow the detailed study of any bacterial taxon in a community if specific primers are used to better understand its ecology (19). In addition, pathogenic bacteria, also unexpected ones, posing a health risk can be observed and identified without their prior anticipation.

16S rRNA-based fingerprint analyses can be based on the analysis of environmental DNA or RNA. In general, it is assumed that RNA-based fingerprints represent the active part, especially the actively growing part, of the bacterial community whereas DNA-based analyses provide insight into the bacterial members present in the community (14, 26). Since viability is a major issue for drinking water bacteria, the comparison of DNA- and RNA-based analyses is of great interest. Combining these DNA- and RNA-based fingerprint analyses with the distinction for membrane integrity was intended to provide new insights in the bacterial microflora and its viability.

Today's drinking water quality assessment is still based on the culture-based detection of indicator bacteria, i. e. *Escherichia coli* or fecal enterococci. Though molecular methods could provide better insights into the bacterial community and increase safety of the drinking water, it is crucial to include the aspect of viability in the molecular methods used. To this end, we developed a procedure that combined the advantages of culture- independent molecular methods and the discrimination of membrane intact and membrane injured cells provided by the viability stains. Using Fluorescence Activated Cell Sorting (FACS), the membrane intact ("live") and membrane injured cells ("dead") were separated and afterwards analyzed by community fingerprinting. The aim of our study was to elucidate by this approach which bacterial taxa are alive in finished drinking water. Both nucleic acids, DNA and RNA, were extracted from three fractions, i.e. total, "live" and "dead", and analyzed by 16S rRNA-based and 16S rRNA gene-based SSCP fingerprinting followed by sequencing of the fingerprint bands to provide insight into the taxonomic composition of the bacterial community. The differences between DNA- and RNA-based fingerprints were analyzed to gain information about the active part of the bacterial drinking water microflora, with the new aspect of membrane integrity. To our knowledge, this is the first study that applies both, DNA- and RNA- based community analysis combined with live/dead staining.

5.3 Results

5.3.1 Bacterial cell counts and heterotrophic plate counts.

The results on the bacterial counts are detailed in Figure 1. For drinking water samples obtained from the tap at the three sampling dates, the total bacterial cell numbers were in the range of 3 to 4 x 10^5 cells ml^{-1}; in the concentrates (100 to 400 fold) of the drinking water bacteria used for viability staining the cell numbers ranged from 5.1 x 10^7 to 1.2 x 10^8 cells ml^{-1}. After staining with PI and SYTO9, the fraction of membrane intact cells determined microscopically accounted for 53% ± 6% of the total bacteria while the membrane injured fraction accounted for 47% ± 6%. Heterotrophic plate counts (HPC) made from the concentrates were on average substantially less than the total bacterial counts, i.e. four to five orders of magnitude depending on medium and incubation time. Heterotrophic plate counts on R2A agar at 22°C and after 72h exceeded all plate counts on the other media and temperatures, and ranged from 2.0 to 4.1 x 10^3 CFU ml^{-1} in the concentrate. For the not concentrated tap water between 3 and 31 CFU ml^{-1} were detected.

5.3.2 FACS results of PI/SYTO stained drinking water bacteria.

After PI/SYTO staining, drinking water bacteria were analyzed based on two scatter parameters (forward and side scatter) and the fluorescence signal. For the analysis, some bacteria were excluded due to a lower forward scatter signal indicating cell debris with little or no DNA content (Figure 5.2a). After staining, the majority (around 70-80%) of all cells could be sorted into two fractions, i.e. non membrane-injured SYTO9 positive cells and membrane-injured PI positive cells (Figure 5.2b). Subsequent purity control as well as a check by epifluorescence microscopy demonstrated the effectiveness of the sorting (Figure 5.2c and d). Flow cytometric analysis of the drinking water bacteria, based on comparison with reference beads of defined sizes, indicated that all fractions of microorganisms (total, SYTO9 positive, PI positive) had a narrow size distribution and a rather small diameter, i.e. on the average 0.69µm (c_v:1.3%) (data not shown). In the three sorting experiments, total cell numbers recovered from FACS ranged around 10^6 cells per fraction (membrane intact, membrane injured) that were subsequently subjected to nucleic acid extraction and fingerprinting.

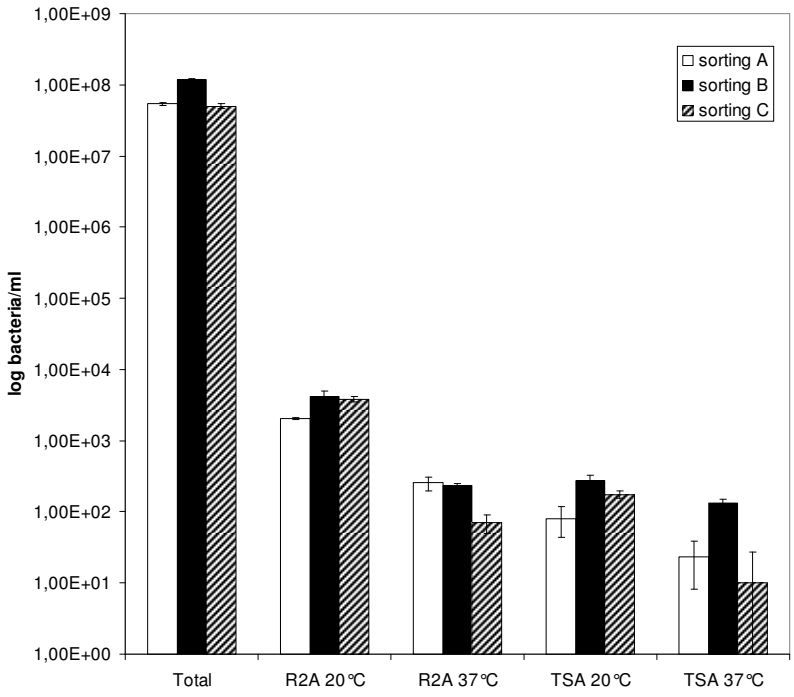

Figure 5.1: Total bacterial cell numbers of the drinking water concentrate used in the three FACS sorting experiments sorting A (25.03.08, open bars) sorting B (31.03.08, black bars) and sorting C (05.05.2008, hatched bars). Total bacterial counts were determined by epifluorescence microscopy using Sybr Green I staining of formaldehyde fixed samples. Heterotrophic plate counts were determined using 1ml (or appropriate dilutions) concentrated drinking water and the spread plate technique on the media and temperatures indicated. Error bars represent standard deviation of at least 3 replicates.

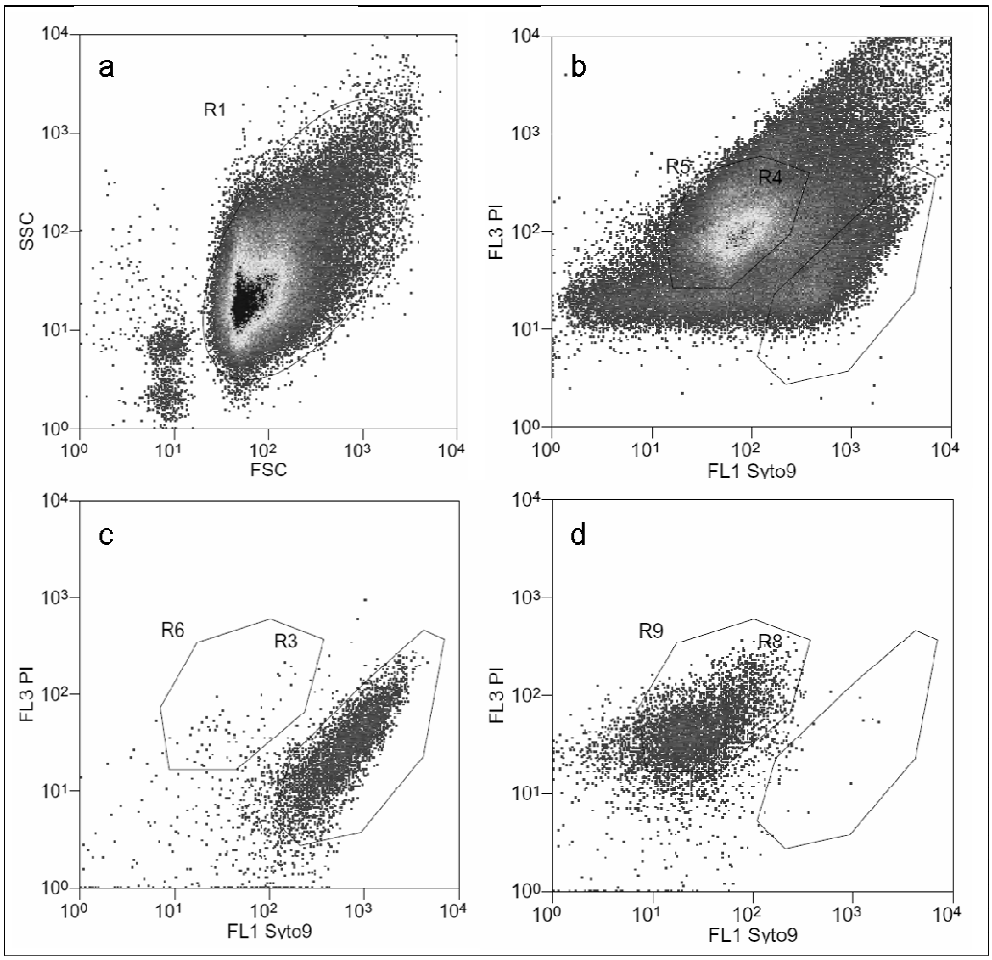

Figure 5.2: Results of the FACS analysis of the drinking water community. Microorganisms from 18 liters of drinking water were concentrated, stained with the BacLight Kit™ and analyzed by the flow cytometer. (a) Flow cytometric analysis of unstained cells. Cells in gate R1 are included in the analysis and cells outside the gate were considered cell debris. (b) Flow cytometric analysis of microorganisms stained with the BacLight Kit™. Cells in gate R4 are Syto 9 positive, cells in gate R5 are PI positive. Purity control of the sorted fractions: (c) Syto 9 positive cells (gate R3) but PI negative (gate R6) and in (d) PI positive cells (gate R9) but negative for Syto 9 (gate R8). Fluorescence channel: FL 1, 530±40nm; FL3, 616±16nm; FSC, forward scatter; SSC, side scatter.

5.3.3 Structure of the bacterial community of drinking water before and after sorting.

DNA- and RNA-based 16S rRNA SSCP fingerprints were used to analyze the bacterial community structure and composition of the drinking water before and after the cells were sorted by FACS as membrane intact and membrane injured cell fractions, and to assess the effect of the concentration procedure on the bacterial community (Figure 5.3). A general observation was that DNA- and RNA- based fingerprints from the same samples showed always very different banding patterns, a feature that was confirmed (see below) by the analysis of the species composition by sequencing of the fingerprint bands. DNA- and RNA-based SSCP fingerprints of the drinking water community with and without concentration (the latter sampled on filter sandwiches) were highly comparable (see Supplementary Material Figure 5.1). Fingerprints of the unsorted drinking water concentrates generated on the three sampling dates clustered closely together indicating a high similarity for the structure of the drinking water bacterial community on the three sampling dates (Figure 5.3, Figure 5.4). As shown in Figure 5.4, the highest similarity was observed among sampling A and B for the DNA-based fingerprints (95%); the similarity of the concentrates was always higher than 76% irrespective of DNA- or RNA-based analyses or the sampling date (Figure 5.4a, b, respectively).

DNA-based fingerprints of the membrane intact and membrane injured sorted fractions showed a very distinct pattern for each sampling day (Figure 5.3a, Figure 5.4a). Comparative cluster analysis of the DNA-based fingerprints showed that for each sampling date the fingerprints from each fraction clustered more closely together than the different sampling dates, indicating that the community structure became more dissimilar among the sampling dates due to the live/dead sorting (Figure 5.4a). Remarkably, after sorting the live and dead fractions of all three samplings were most closely related to each other indicating that the DNA-based fingerprints reflected the same live and dead bacteria (phylotpyes). In contrast, the RNA-based fingerprints of the sorted cell fractions showed a similar pattern among the membrane intact fractions irrespective of the sampling date (Figure 5.3b, Figure 5.4b) as indicated by a tight clustering (similarity >70%, Figure 5.4b). The membrane injured sorted fractions showed a more diverse pattern for the three sampling dates, mainly caused by the large discrepancy for sampling C.

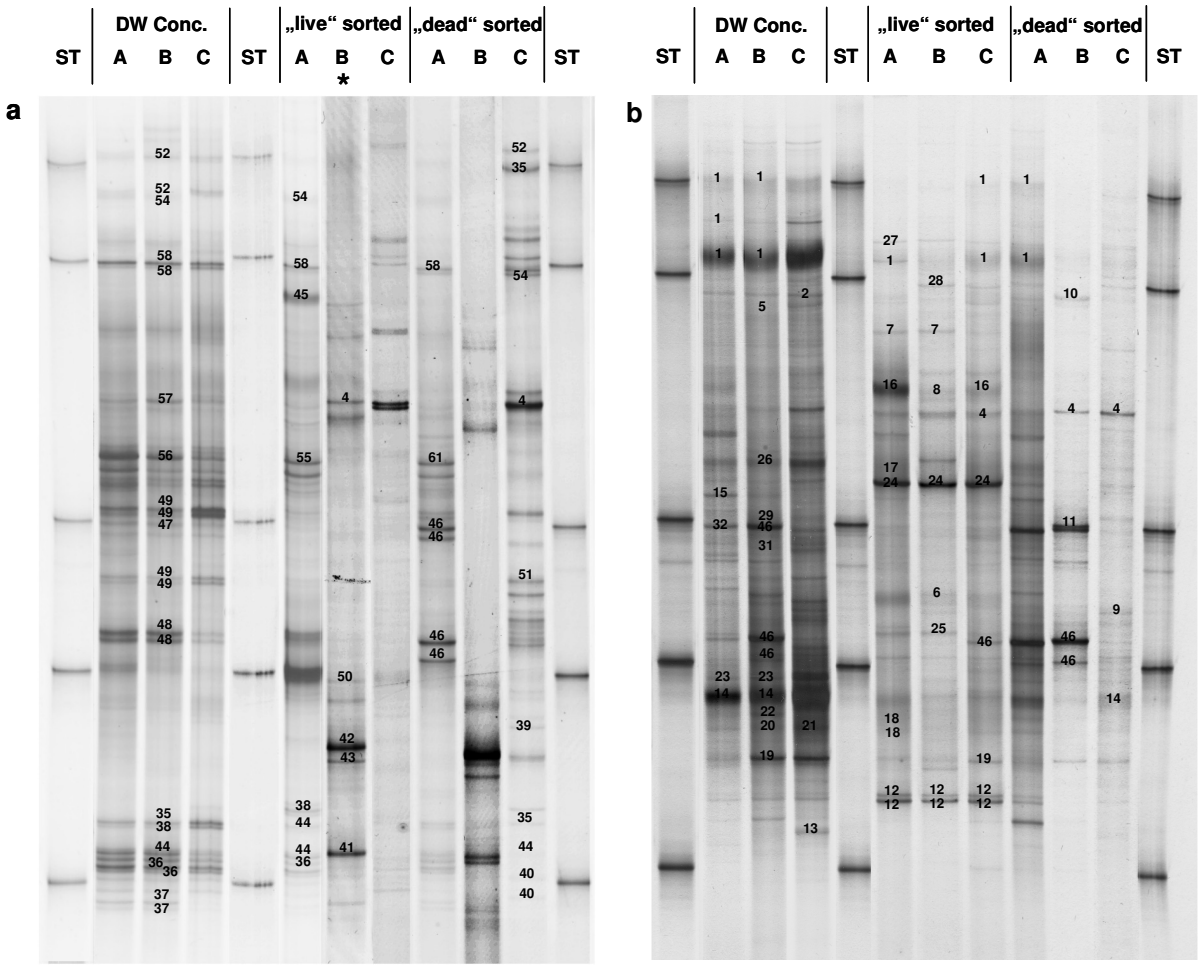

Figure 5. 3: (a) DNA-based 16S rRNA gene SSCP fingerprints of the different FACS sorting experiments: sorting A (25.03.08) sorting B (31.03.08) and sorting C (05.05.2008). Numbers represent single phylotypes of sequenced and identified bands. Phylogenetic information about these phylotypes is given in Supplementary Material Table 5.1. Designations of single samples: ST, species standard; DW Conc., concentrated, unsorted drinking water samples from the respective dates; membrane intact ("live") sorted, SYTO9 positive fraction of the drinking water; membrane injured ("dead") sorted, propidium iodide positive fraction of the drinking water. The asterisk indicates a lane from a different SSCP gel. (b) RNA-based 16S rRNA SSCP fingerprints of the different FACS sampling dates. Sample designations and numbering of sequenced phylotypes are as for panel a. Phylogenetic information about the numbered phylotypes is given in Supplementary Material Table5. 2.

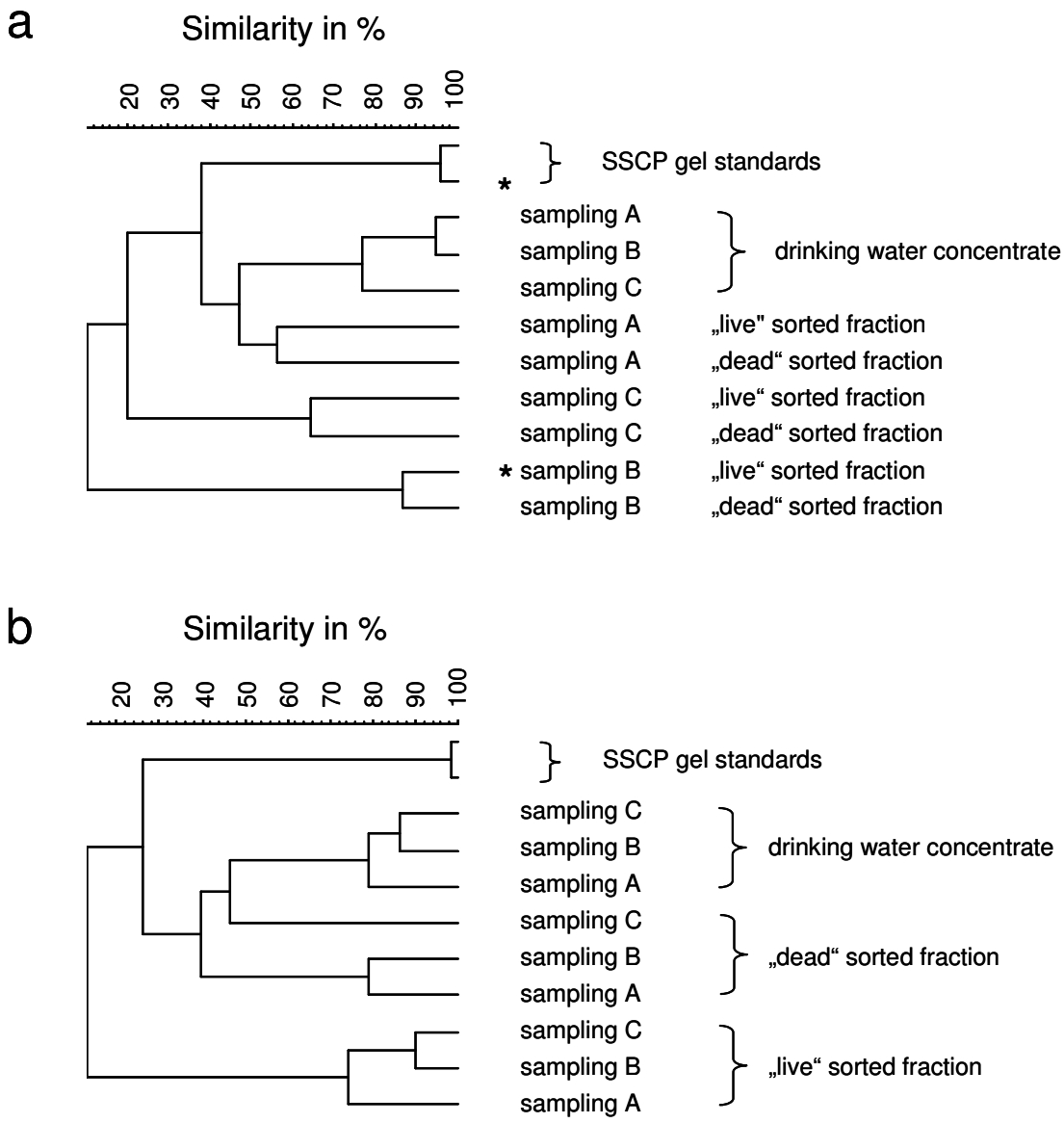

Figure 5.4: Cluster analysis of the two SSCP gels given in Figure 3. Similarity coefficients were calculated using Pearson correlation algorithm. Dendrograms were constructed using the Unweighted Pair Group Method with Arithmetic mean. (a) DNA-based SSCP fingerprints of the different FACS sampling dates: sorting A (25.03.08) sorting B (31.03.08) and sorting C (05.05.2008). Sample designations are as in Figure 5.3a. The lane labeled with an asterisk is from a different SSCP gel. (b) RNA-based SSCP fingerprints of the different FACS sampling dates. Species standards were taken as out-group for the cluster analysis. Sample designations are as in Figure 5.3b.

5.3.4 Taxonomic composition of the different cell fractions.

A total of 111 bands from the DNA- and RNA-based SSCP fingerprints were sequenced to determine the taxonomic composition of the different fractions. Using a limit of ≥ 99% 16S rRNA gene sequence similarity as discrimination criterion, we retrieved 53 unique phylotypes for these bands (Supplementary Material Table 5.1 and 5.2). For identification, the obtained sequences were compared to all databank entries in the GenBank. Out of the 53 unique phylotypes, 31 were retrieved from the RNA-based fingerprints, and 24 from the DNA-based fingerprints with only two phylotypes that were retrieved from both RNA and DNA. RNA-phylotype 1 and DNA-phylotype 52 were affiliated with the same species but were distinct by 8 nt and therefore assigned to different phylotypes. Thus, the bacterial community reflected by both fingerprint types differed to a large extent.

Comparing the major taxonomic groups, the analysis of the DNA-based fingerprints (Table 5.1, Figure 5.5a,) showed that the drinking water samples were dominated by members of the *Betaproteobacteria* (8 phylotypes, with an average abundance of 14.9%), *Bacteroidetes* (7 phylotypes, 17.8%), and *Actinobacteria* (2 phylotypes, 15.3%). All other classes and phyla, i.e. *Alpha-* and *Gammaproteobacteria*, *Planctomycetes* and *Cyanobacteria*, had a low diversity (1-2 phylotypes) and a low abundance (0.2 - 3.3%). The RNA-based fingerprints (Figure 5.5b) of the drinking water samples were dominated by members of the *Betaproteobacteria* (4 phylotypes, 20.8%), *Cyanobacteria* (6 phylotypes, 15.6%), *Alphaproteobacteria* (5 phylotypes, 15.5%), *Gammaproteobacteria* (8 phylotypes, 9.5%), and *Bacteroidetes* (3 phylotypes, 8.3%). The remaining 4 phyla, i. e. *Nitrospira, Firmicutis, Planctomycetes, Chloroflexi*, had a low diversity (1-2 phylotypes) and a low abundance (0.1-4.6%). While most phyla occurred in both the RNA- and DNA-based analyses, *Actinobacteria* were never observed in the RNA-based analyses, whereas *Chloroflexi* (with a high abundance of 16% in the membrane intact fraction of the RNA-based analyses) were never observed in the DNA-based analyses (Table 5.1, Supplementary Material Table 5.1). The single phylotypes of *Nitrospira* and *Firmicutes* also occurred only in the RNA-based analyses but had low and variable abundances (below 2.3%).

Table 5.1: Abundances of the phylotypes (PT) summed up per Phyla/class displayed for the unsorted and sorted ("live/dead") fractions. The abundances of the phylotypes are derived from the SSCP analyses of DNA and RNA extracts as shown in Figure 5.3 (for details on the abundances of the single phylotypes see Supplementary Table 5.2). The mean of the three samplings A,B and C plus the standard deviation SD is given for the tap water sample before sorting ("All (unsorted)"), the "live" sorted fraction (cells with intact membranes), and the "dead" sorted fraction (cells with injured membranes).

DNA based analysis		**All (unsorted)**		**Live**		**Dead**	
Phyla/Class	**PT(n)***	**mean A-C**	SD	**mean A-C**	SD	**mean A-C**	SD
Alphaproteobacteria	1	2.83%	2.24%	1.56%	1.48%	0.92%	1.49%
Betaproteobacteria	8	14.93%	0.52%	16.82%	6.55%	9.92%	10.80%
Gammaproteobacteria	2	0.34%	0.59%	0.50%	0.86%	1.05%	1.81%
Actinobacteria	2	15.32%	1.12%	4.52%	3.71%	5.68%	6.11%
Bacteroidetes	7	17.75%	4.56%	15.45%	22.67%	8.49%	3.02%
Cyanobacteria	2	3.30%	0.97%	0.64%	1.10%	10.10%	17.49%
Planctomycetes	2	0.22%	0.38%	12.81%	22.19%	n.d.	
identified as PTs	24	**54.69%**	3.83%	**52.29%**	21.52%	**36.16%**	23.66%
% PTs with only "dead" cells		**20.8%**					

RNA based analysis		**All (unsorted)**		**Live**		**Dead**	
Phyla/ Class	**PT(n)***	**mean A-C**	SD	**mean A-C**	SD	**mean A-C**	SD
Alphaproteobacteria	5	15.49%	11.41%	21.04%	20.67%	11.52%	8.19%
Betaproteobacteria	4	20.76%	5.66%	9.28%	3.14%	10.33%	4.27%
Gammaproteobacteria	8	9.50%	4.02%	17.58%	5.60%	14.27%	11.31%
Bacteroidetes	3	8.31%	2.64%	2.49%	2.31%	2.49%	3.96%
Chloroflexi	2	0.70%	0.47%	15.75%	9.10%	3.28%	2.13%
Cyanobacteria	6	15.59%	7.56%	7.85%	7.33%	16.23%	8.13%
Firmicutes	1	2.25%	1.95%	0.95%	1.64%	n.d.	n.d.
Nitrospira	1	0.10%	0.17%	n.d.		1.82%	1.84%
Planctomycetes	1	4.60%	7.96%	n.d.		n.d.	
identified as PTs	31	**77.30%**	3.38%	**74.93%**	20.17%	**59.94%**	5.68%
% PTs with only "dead" cells		**21.4%**					

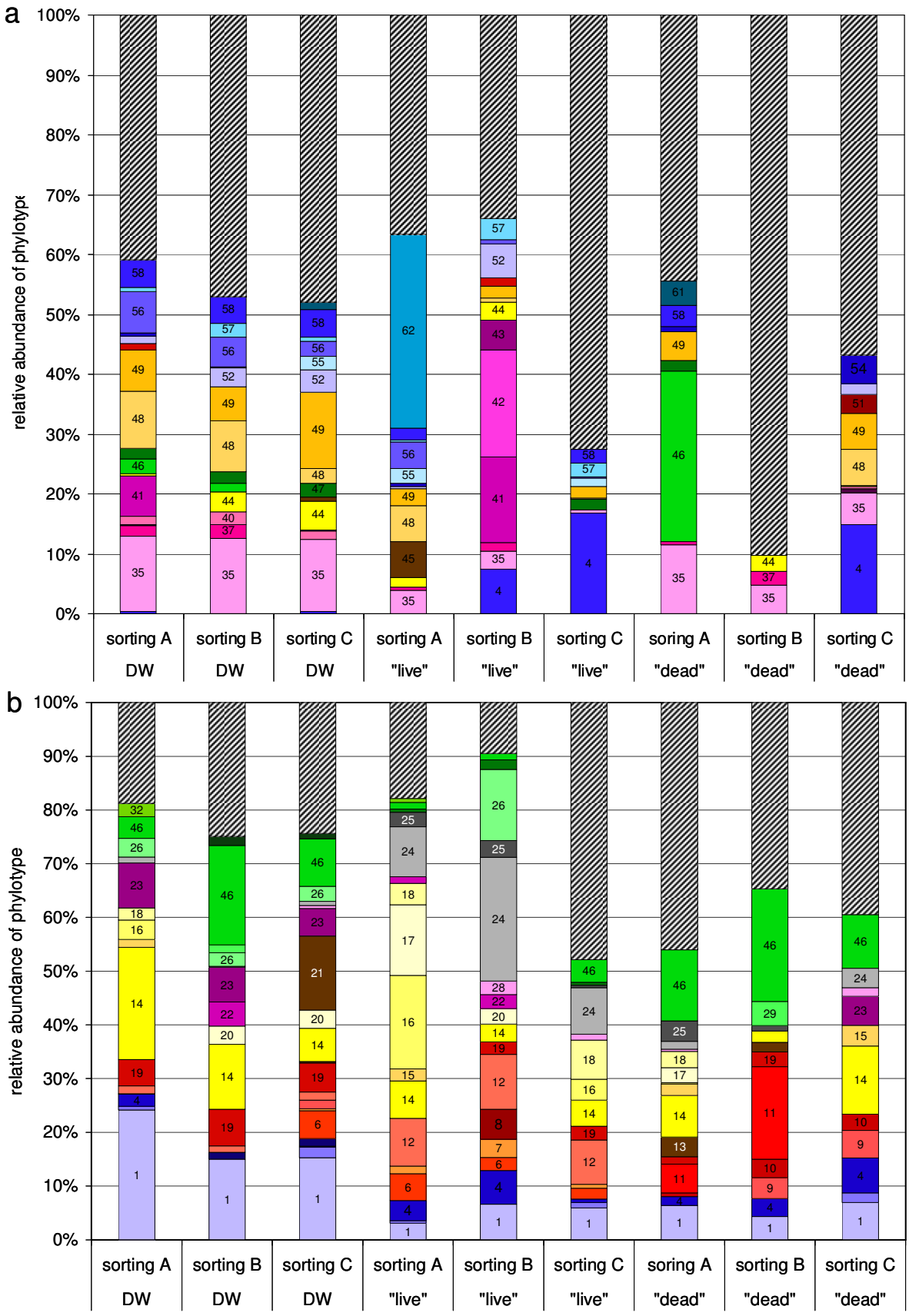
a
b
relative abundance of phylotype
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
sorting A DW
sorting B DW
sorting C DW
sorting A "live"
sorting B "live"
sorting C "live"
soring A "dead"
sorting B "dead"
sorting C "dead"

Figure 5.5: Comparison of relative abundances of the phylotypes found in the different cell fractions and the drinking water concentrate (DW) on the three different sampling dates. (a) Phylotypes from the DNA-based SSCP fingerprints. (b) Phylotypes from the RNA-based SSCP fingerprints. Numbers represent the single phylotypes given in Supplementary Material Table 5.1 and 5.2, respectively. The colors are corresponding to the major phylogenetic groups of the phylotypes: Yellow – Alphaproteobacteria; Blue – Betaproteobacteria; Red – Gammaproteobacteria; Green – Cyanobacteria; Violet – Bacteriodetes; Brown – Planctomycetes; Orange – Actinobacteria; Grey – Chloroflexi. Hatched bars represent unidentified bands.

An overview on the phylogenetic diversity is shown by Supplementary Figure 3a by a tree based on the phylogenetic analysis of the retrieved phylotypes together with the nearest cultured species. Supplementary Figure 3a shows all occurring phyla and Figure 5.3b shows the phylum *Proteobacteria* in more detail. Details of the phylogenetic analyses are listed in Supplementary Table 5.1 and 5.2. Overall, the bacterial drinking water community retrieved from RNA and DNA analyses was mostly composed of bacteria that were not related to any described species. For the DNA-based analyses 46% of the phylotypes were not related to any described genus, 42% were affiliated with a described genus, and 38% were affiliated with a described species. For RNA-based analyses 58% of the phylotypes were not related to any described genus, 32% were affiliated with a described genus, and 23% were affiliated with a described species. The phylotypes affiliated with a described genus were mostly members of the *Bacteroidetes, Alpha-, Beta-* and *Gammaproteobacteria*.

From the 24 phylotypes of the DNA analyses, three phylotypes contributed to more than 5% (up to 12%) of the total (unsorted) drinking water community (Supplementary Material Table 5.1 & 5.2). Two of these three dominating phylotypes were related to uncultured *Actinobacteria* (phylotype 48, 49). The bacterium with the highest abundance of 12.4% showed 98% similarity to the freshwater bacterium *Sediminibacterium salmoneum*, a cultured *Bacteriodetes* (phylotype 35). From the 31 phylotypes of the RNA analyses, five phylotypes contributed to more than 5% (up to 18%) of the total (unsorted) drinking water community. These five dominating phylotypes were composed of one cyanobaterium (phylotype 46; affiliated with the genus *Synechococcus*), one gammaproteobacterium with related only to uncultured bacteria (phylotype 19), one betaproteobacterium related to the species *Acidovorax*

facilis (phylotype 1), one alphaproteobacterium related to the species *Bosea vestrii* (phylotype 14), and one member of the *Bacteroidetes* (phylotype 23) not related to any described genus.

All 24 DNA-based phylotypes were recovered after cell sorting in the membrane intact and/or membrane injured fractions indicating a recovery of 100% of the phylotypes in the sorted fractions. 38% of the DNA-phylotypes occurred only in the membrane intact fraction, 21% occurred only in the membrane injured fraction, and 42% occurred in both fractions. Phylotypes of the major taxa *Betaproteobacteria* and *Bacteroidetes* contributed to all three fractions, i.e. membrane intact, membrane injured and total. The two phylotypes of the *Actinobacteria* were always retrieved from the membrane intact and membrane injured fractions. Based on the RNA analyses, 28 of the 31 phylotypes (90%) were retrieved after sorting in the membrane intact and/or membrane injured fraction. From the retrieved 28 phylotypes, 32% of the RNA-phylotypes occurred only in the membrane intact fraction, 21% occurred only in the membrane injured fraction, 46% occurred in both fractions. Phylotypes of the classes *Gammaproteobacteria*, *Cyanobacteria* and the phylum *Bacteroidetes* contributed to all three fractions, i.e. membrane intact, membrane injured and total. All phylotypes of the *Alphaproteobacteria* were always retrieved from membrane intact and membrane injured fractions. Thus, the phylotypes obtained from RNA- and DNA-based analyses showed a similar ratio with respect to retrieval of their cells from the membrane intact and injured fractions: 32-38% had cells only in the membrane intact fractions, 21% only in the membrane injured fractions, and 42%-46% in both fractions.

After FACS-sorting, major changes of the abundances of the phylotypes occurred that were far more pronounced for the DNA-based analyses than for the RNA-based analyses. Supplementary Table 5.1 and Supplementary Figure 5.2 are providing the details on the changes of abundances with respect to the phylotypes before and after sorting, while Table 5.1. provides an overview on the phyla/class level. These changes of abundances through sorting were most pronounced in the membrane intact sorted fraction for the *Chloroflexi* (PT 24) in the RNA- based analyses and the *Planctomyces* (PT 62) in the DNA-based analyses. Overall, we observed only few phylotypes with a high abundance in the sorted cell fractions of the DNA-based electropherograms (Supplementary Material Figure 5.2a) while in the RNA-based electropherograms (Supplementary Material Figure 5.2b) phylotypes with a high abundance were present in the non-sorted as well as in the sorted fractions.

For an estimate of the origin of the phylotypes, the habitat of the most similar bacterial sequence from the public data bases is given in Supplementary Table 5.1 and 5.2. Provided that the most similar sequence i) had a similarity of higher or equal to 91% 16S rRNA gene similarity and ii) was of aquatic origin, the phylotype was rated as "of aquatic origin". Below 91% 16S rRNA gene sequence similarity the relatedness was regarded as too low to give information on the potential habitat of the phylotype. Based on these criteria, 76% of the DNA and the RNA-based phylotypes were considered as of aquatic origin which most of them from freshwater habitats. Six out of the RNA phylotypes and three out of the DNA phylotypes were not used for this assignment due to too low 16S rRNA gene sequence similarity (all these sequences had a similarity below 88% to the next sequence in the public data bases).

5.4 Discussion

5.4.1 Community structure and composition of drinking water bacteria using DNA- and RNA-based fingerprints.

DNA- and RNA-based molecular analyses provided a very different picture of the drinking water microflora. This comprised the overall fingerprint patterns, their changes due to sorting and the retrieved phylotypes. However, this overview does not precisely reflect the quantitative composition of the bacterial community. Since the amplification of 16S rRNA genes is based on PCR, a PCR bias has to be taken into account (16, 36). According to our experience with aquatic community analysis by SSCP, the technique provides highly reproducible fingerprints of the community with high reproducibility in terms of the relative abundances of the single bands compared to the total community. Compared to real-time PCR detection of single phylotypes, low abundant phylotypes seem to be overestimated, while highly abundant phylotypes seem to be underestimated (8). Thus, the fingerprint gives a biased but reproducible semi-quantitative picture of the bacterial community allowing comparison of different bacterial communities and observation of the dynamics of single community members.

The fingerprint analysis of the drinking water samples showed a highly consistent pattern among the three different sampling dates for both the RNA- and DNA-based analyses. A rather stable bacterial community of the investigated drinking water over time had already been shown by the seasonal study of Henne et al. (19), using DNA-based fingerprints. Though seasonal variation occurred for some members of the bacterial community, the overall community structure was rather stable during the year. The SSCP fingerprint patterns were completely different with respect to analysis of RNA and DNA of the same samples. This different pattern was confirmed by sequencing and phylogenetic analysis of the fingerprint bands. From the 24 phylotypes retrieved from the DNA-based analysis, and 31 phylotypes retrieved from the RNA-based analysis only two phylotypes (PT 4, 46) were identical, and two were affiliated with the same species (PT 1, 52). Though the same phyla with a few exceptions were detected in RNA- and DNA-based analysis, from the genus level upwards there was a pronounced divergence at the species level. This strong discrepancy between RNA and DNA-based analysis concerning the fingerprint pattern and the members of the bacterial community had already been observed by Eichler et al. (14).

Our drinking water community was dominated by phyla and classes typical for freshwater environments, i.e. *Bacteroidetes, Cyanobacteria, Betaproteobacteria* and *Gammaproteobacteria*. This was also the case when looking at the higher level of phylogenetic resolution, i.e. the phylotypes that were resolved approximately at the species level. The majority of the phylotypes (76%) were most closely related to sequences retrieved from aquatic habitats. This is consistent with findings of the study of the whole drinking water supply system by Eichler et al. (14). The phylotypes identified based on the DNA-based analyses seemed to have a higher stability in the drinking water than the RNA phylotypes. 55% of the DNA-phylotypes identified in this study were also detected in the study of Eichler et al. in the same drinking water supply system 5 years ago. This was different for the RNA-based phylotypes that had only a reoccurrence of 11%.

5.4.2 Assessment of live and dead bacterial cells using PI/SYTO9 staining.

In our study about half (53%) of the bacterial cells in the drinking water samples showed an intact membrane. This is in line with studies by Berney et al. that reported a fraction of membrane intact cells of about 66% in tap water that was free of chlorine as it was the case in our study (4). For chlorine containing tap water, Hoefel et al. reported 12% membrane intact cells for finished drinking water of an Australian water distribution system with a higher chlorination during treatment and transport, and a free chlorine residual level of 0.4 mg l^{-1} at the tap (20).

The Propidium Iodide staining is considered to provide a good estimate for membrane injury of *Bacteria* and *Archaea* (24). In a set of studies, this staining procedure has been evaluated and compared with other staining procedures for assessment of the physiological state of the bacteria (15, 22). Besides the evaluation of methodological aspects, recently studies were done for drinking water with added bacteria and the indigenous microflora. Berney et al. tested PI for *E. coli* in drinking water submitted to UV and sunlight irradiation using a set of different viability stains (3). The study showed that loss of membrane integrity as indicated by PI staining was the final signal after decrease of all other tested physiological functions. In a second study, Berney et al. used PI staining for analyzing the microflora of a set of drinking water samples (4). The viability of the drinking water bacteria was higher for bottled

water (about 90%) and drinking fountain water (about 85%) than for drinking water at the tap (about 66%). The high percentage of viable cells coincided with a high ATP content. The comparison of PI staining with other methods demonstrated PI staining was a valuable criterion for live-dead distinction for drinking water bacteria.

Autofluorescence is a feature that has to be taken into account as a potentially misleading signal for the analysis of aquatic bacterial communities by PI/SYTO9 staining (37). According to our taxonomic analyses, two phylotypes were affiliated with the phylum *Chloroflexi* whose members are known to contain bacteriochlorophyll c and a in the chlorosomes and the cytoplasmic membrane resulting in green autofluorescence (25). The *Chloroflexi* were detected in the membrane intact and membrane injured sorted fractions, but with a far higher detection in the membrane intact fractions (up to 23% for PT 24 in the RNA-based analyses). In the latter case a wrong "live" sorting due to the autofluorescence cannot be ruled out. On the other hand, a false "dead" sorting could have been caused by phylotypes affiliated with the genus *Synechococcus* due to the presence of red fluorescent phycoerythrin (35). Phylotype 46 that was common in the RNA- and DNA-based analyses and closely related to *Synechococcus rubescens* had a high abundance in the "dead"- sorting of 10% for the DNA and of 15% for the RNA based analysis, respectively. Though autofluorescence may be misleading for the live-dead sorting of some bacteria with photosynthetic pigments, we do not consider this as a critical issue for the live/dead staining procedure as a distinction for drinking water bacteria. Autofluorescent bacteria are commonly not considered as pathogenic and therefore, autofluorescence does not seem a critical issue for our staining procedure in terms of public health.

5.4.3 Live and dead assessment of different phyla and phylotypes

All DNA-based phylotypes and 90% of the RNA-based phylotypes were retrieved after sorting in the membrane intact and/or membrane injured fraction. The three missing RNA phylotypes might have been missed due to their low abundance in the tap water. This close to complete recovery of the phylotypes after sorting allows a comparison of the sorting results between the DNA- and RNA-based analyses. Though the sequencing success was 77% for the RNA-based analyses, and only 57% for the DNA-based analyses, the comparison can be done on the level of the retrieved phylotypes that indeed had a relatively high abundance compared to the not retrieved phylotypes.

A comparison shows that the phylotypes of the DNA-based analyses had the same size of the "dead fraction" as those reflected by the RNA-based analyses, i.e. 21%. Also, the DNA- and RNA-phylotypes had a comparable percentage of only "live" sorted (DNA, 38%; RNA, 32%) and of "mixed" sorted phylotypes (DNA: 42%, RNA, 46%). Phylotype 4 concomitantly retrieved from DNA- and RNA- analyses was recovered from membrane intact and membrane injured fractions in the DNA- and RNA-based analysis, i.e. for the only common phylotype comparable sorting results were obtained for the DNA and RNA-based analysis. The second common phylotype (PT 46) cannot be compared due to the potential interference with the pigments (see above). Based on our observation, we can say that the fraction of phylotypes with only membrane injured cells is not higher for the bacteria reflected by the DNA analyses than those of the RNA analyses. This is an essential finding because it was often assumed that those reflected by the RNA are alive, and those reflected by the DNA are dead (14). Based on this observation, we assume that the reason for the detection of a phylotype in the DNA- or RNA-based analyses might be the phylotype-specific regulation of the DNA and the RNA pool and was obviously not related to the viability of the respective phylotypes. This is consistent with analyses of Klappenbach et al. showing a broad range of numbers of rRNA operons (1-13) specific for each bacterial strain (23). On the other hand, we observed that all fingerprints of the membrane intact fractions showed rather similar RNA-based fingerprints reflecting actively growing members of the community. This tight clustering of the RNA-based fingerprints from live bacteria could indicate that always the same actively growing members of the drinking water community re-grew after chlorination had killed most of the bacteria in the waterworks. This is no contradiction to the detection of a substantial amount of RNA-based phylotypes in the dead fraction because several of the live RNA-phylotypes were different from the dead ones (phylotype 12, 24) or were abundant in different amounts (Figure 5.5b, Supplementary Table 5.2b). These dead RNA-phylotypes could still be remnants of the highly active phylotypes before chlorination which have not re-grown. Overall, we think that the combination of FACS sorting and fingerprinting is a new way to obtain functional fingerprints – with the live RNA phylotypes representing the most actively growing members of the microbial community (12, 27).

5.4.4 Taxonomic composition of the bacterial community of drinking water and human health

The bacterial community was composed of seven phyla (see Supplementary Material Table 5.1 and 5.2). The phyla as well as the phylotypes are primarily those typically present in aquatic ecosystems (14, 38). However, one phylotype detected in the drinking water had the potential of being an opportunistic pathogen. The alphaproteobacterium PT 14 identified as closely related to *Bosea vestrii* in the RNA-based analysis was retrieved from the membrane intact and membrane injured sorted fraction, and was present in the drinking water at a high abundance of 13%. This species was occasionally associated with infections of immuocompromised people (31).

However, the mere detection of a bacterium at a taxonomic resolution close to the species level is not sufficient as an indication of a health risk. Presence, viability and infectivity of pathogenic bacteria in drinking water are criteria that have to be fulfilled for assessing a threat to human health. Presence of bacteria can be assessed by the applied technology to the detection limit of the method which is about 0.1% of the total microflora. Viability was assessed by the live/dead staining. Infectivity asks first for the precise taxonomic identification of the pathogen and a separate, mostly experimental, assessment of infectivity that has to be achieved in addition to molecular analyses. Concerning the precise assessment of the taxonomy, the about 400nt long sequences obtained from a SSCP gel can resolve, at best, the species level. Though this accuracy might be highly valuable for the study of environmental bacteria, for most pathogenic bacteria, a full (>1400nt) 16S rRNA sequence is needed or even the sequence of other genes associated with infectivity of the respective species, e.g. the *mip* gene for *Legionella pneumophila*. Thus, the proposed technology can provide a valuable monitoring tool that can show that a potentially harmful species is present - but it remains with the “potential” and the true risk has to be assessed consecutively by additional adequate measurements.

In conclusion, the approach used in this study is considered a valuable tool for drinking water monitoring. The applied PI/SYTO9 staining procedure indicating membrane injury of the bacterial cells is considered as a reliable criterion for damaged or dead bacterial cells. This is especially of value for monitoring of bacteria relevant to human health. The combined approach of DNA- and RNA-based fingerprint analyses with live-dead staining and sorting was demonstrated as a straight forward monitoring tool. This tool still can be modified and extended with respect to sensitivity or

methodological details. For example, in terms of methodology, PI/SYTO9 stain could be replaced by propidium monoazide application thereby avoiding the step of FACS sorting (29). On the other hand, the sorted cells can be submitted to further labelling/staining and subsequent analyses. For increased sensitivity with respect to specific groups of pathogenic relevance, the general bacterial primers (COM1, 2) could be replaced by group specific primer reaching a lower detection limit and a better taxonomic resolution of the targeted group.

From an ecological perspective, the study provided comprehensive insights into the community composition and its viability of drinking water bacteria and shows that a very different spectrum of species was detected by DNA- and RNA-based analysis. A major finding in ecological terms is the fact that the viability of the phylotypes was comparable for RNA and DNA extracts. The viability of the phylotypes in addition to the very different spectrum of species detected (included pathogenic ones) demonstrate the value of adding RNA-based analyses to the commonly applied DNA-based analyses for drinking water studies or, in more general terms, for aquatic studies.

5.5 Experimental procedures

5.5.1 Study site and sampling.

Drinking water samples were obtained on 3 days, i.e. 25 March 2008 (sampling A), 31 March 2008 (sampling B) and 5 May 2008 (sampling C) from the tap in lab D0.04 of the Helmholtz Centre for Infection Research (HZI), Braunschweig-Stöckheim, Germany. Sampling A and B were taken as samples where a high similarity was expected due to the short time interval, sampling C was considered to display a distinct community due to the previously observed seasonal changes (19). The drinking water originated from two surface water reservoirs (oligotrophic, and dystrophic water) situated in a mountain range 40 km south of Braunschweig. Water processing included flocculation/coagulation, sand filtration and chlorination (0.2 - 0.7 mg l^{-1}). In 2008 and 2009 no chlorine was detected at the nearest sampling point upstream to the HZI by the local water supplier by the colorimetric test "Aquaquant Chlor" from Merck for detection of free and total chlorine (detection limit 0.01mg/l). More details on the respective drinking water supply system are given elsewhere (14).

For live/dead staining and fluorescence activated cell sorting (FACS), drinking water microorganisms were concentrated 100-400 fold. 18 liter of drinking water were filtered onto a 0.2 µm pore size polycarbonate filter (90-mm diameter; Nucleopore; Whatman, Maidstone, United Kingdom), scraped and washed off from the filter carefully with 25 ml of 0.9% NaCl in sterile water (Figure 5.6). A part of the biomass was either immediately used for the staining procedure as indicated below, and an aliquot was immediately frozen for later molecular analysis (-70°C).

For comparing the impact of concentration on the drinking water microflora, the drinking water microorganisms were additionally harvested by our routine procedure, i.e. filtration of 5 liters of drinking water on a filter sandwich consisting of a 0.2 µm pore size polycarbonate filter (90 mm diameter; Nucleopore; Whatman, Maidstone, United Kingdom) with a precombusted glass fiber filter on top (90 mm diameter; GF/F; Whatman) according to Eichler et al. (13). Filter sandwiches were stored at -70°C until further analysis. Per sampling date, 5 sandwich filters were obtained.

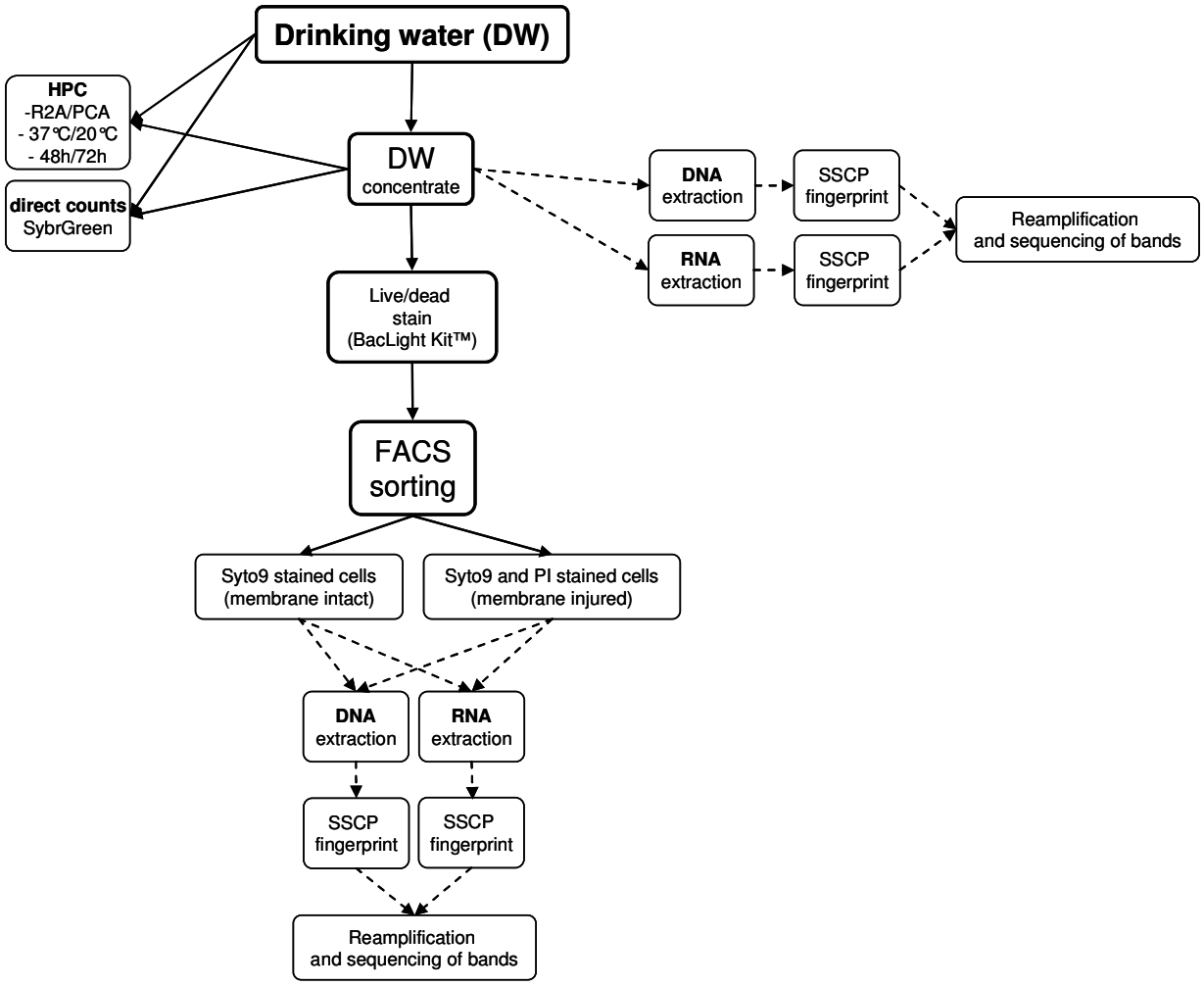

Figure 5.6: Flow chart of the combined analysis of drinking water samples using FACS and SSCP fingerprinting. 18 liters of drinking water were filtered onto a 0.2µm Nucleopore filter, scraped and washed off the filter with 0.9 % saline solution. The drinking water bacteria were stained with the BACLight Kit™ for 20 min in the dark. After cell sorting, the differently stained fractions were analyzed by molecular methods (dashed lines), i.e. nucleic acids (DNA and RNA) were extracted and subjected to SSCP analysis. Sequence information was gained by reamplification and sequencing of single bands.

5.5.2 Staining and enumeration of drinking water bacteria.

Total bacteria from formaldehyde-fixed samples (2% final concentration) were stained with Sybr Green I dye (1:10000 final dilution; Molecular Probes, Invitrogen) for 15min at room temperature in the dark. Five ml portions were filtered onto 0.2 µm pore size Anodisc filters (Whatman) and mounted with Citifluor on microscopic glass slides according to Weinbauer et al. (33). Slides were either analyzed directly with

epifluorescence microscopy or stored frozen (-20°C) until examination. For epifluorescence microscopy, a microscope (Axioplan, Zeiss) with suitable fluorescence filters was used and the slides were examined using 100fold magnification. For each filter, either 10 photographs were taken and image sections of defined size (0.642mm x 0.483mm) were analyzed using the Image J software from MacBiophotonics (http://www.macbiophotonics.ca/) or 30 fields (0.125mm x 0.125mm) were counted by eye.

5.5.3 Heterotrophic plate counts (HPC).

HPCs were done in triplicate using an aliquot of the drinking water concentrate and the spread plate technique on either R2A agar (Oxoid) or tryptone soy agar (TSA; Oxoid) plates. Incubation was carried out at two different temperatures according to the German drinking water ordinance (36°C for 48h and 22°C for 72h) (9).

5.5.4 Concentrating, live/dead staining and FACS analysis of drinking water bacteria.

For fluorescence activated cell sorting (FACS), the concentrated biomass of the drinking water samples was stained for subsequent FACS analysis with SYTO 9 and propidium iodide (PI, final concentrations 5µM and 30µM, respectively; BacLight Kit, Molecular Probes (18)) according to the prescription of the manufacturer. After an incubation time of 20min in the dark, cells were subjected to FACS sorting using a MOFLO cytometer (Beckman Coulter, Krefeld, Germany) with a 488nm laser. The band pass filters used were 530/40nm and 616/26nm for SYTO 9 and PI, respectively.

5.5.5 Nucleic acid extraction from drinking water and sorted fractions.

DNA- and RNA- were extracted from the filter sandwiches and the concentrates of the drinking water samples; the latter were analyzed before and after staining and FACS-sorting as described above. For extraction of DNA and RNA, a modified DNeasy/RNeasy protocol (Qiagen, Hilden, Germany) was used. In this procedure, sandwich filters were cut into pieces, incubated with lysis buffer containing 10mg/ml lysozym (Sigma) for 30 min (DNA) or 20 min (RNA) in a 37°C water bath. After a

mechanical homogenization by shaking with glass beads the samples were heated to 70°C in a water bath for 20min (DNA) or 15min (RNA). After filtration through a polyamide mesh with 250µm pore size, absolute ethanol was added to the filtrate (ratio filtrate/ethanol 2:1) and the mixture was applied to the adequate spin-column of the kit. After this step, the protocol was applied according to the manufacturer's instructions. For the RNA, a subsequent on-column DNase digestion was applied. Nucleic acids were eluted from the columns with DNase/RNase free water and stored at -20°C. The nucleic acids were quantified using Ribogreen (RNA or ssDNA quantification, Molecular Probes; Invitrogen) or Picogreen (dsDNA quantification, Molecular Probes; Invitrogen) according to Weinbauer et al. (34).

For extraction of the nucleic acids from the concentrated or the sorted fractions of microorganisms (considered as dead or alive), 1-2 ml portions of the concentrates before and after sorting were harvested by centrifugation for 15min at 15.000xg. The pellets were either frozen or directly used for nucleic acid extraction using the DNeasy/RNeasy protocol (Qiagen, Hilden; Germany). Pellet supernatant was checked by epifluorescence microscopy for microorganisms; in no case cells were observed. DNase digestion for the RNA was applied as described above.

5.5.6 16S rRNA and 16S rRNA gene based community fingerprints.

PCR amplification of 16S rRNA and of its respective genes from the extracted nucleic acids were performed using the previously described primers COM1 (5´-CAGCAGCCGCGGTAATAC-3´) and COM2 (5´-CCGTCAATTCCTTTGAGTTT-3´), amplifying positions 519 to 926 of the *Escherichia coli* numbering of the 16S rRNA gene (30). For single strand separation a 5´-biotin-labeled forward primer was used according to Eichler et al. (14). From RNA, a reverse transcription was carried out before PCR using the First strand cDNA synthesis Kit (Fermentas) following the manufacturer's recommendations. Each amplification was carried out using 2 ng DNA/cDNA template in a final volume of 50 µl, starting with an initial denaturation for 15 min at 95°C. A total of 30 cycles (30s at 95°C, 30s at 55°C, and 1 min at 72°C) was followed by a final elongation for 10 min at 72°C. Amplification was achieved using HotStarTaq DNA polymerase (QIAGEN, Hilden, Germany).

For the preparation of ssDNA and community fingerprints, a variant of the protocol described by Eichler et al. was applied (14). Briefly, magnetic streptavidin coated beads (Promega, Madison, Wis.) were applied to obtain ssDNA from the PCR amplicons.

Quantification of the obtained ssDNA was performed on a 1.5% agarose gel by comparison with a low-molecular-weight marker (Invitrogen low-DNA-mass ladder). For SSCP fingerprinting analysis, 25 ng of the obtained ssDNA was mixed with gel loading buffer (95% formamide, 10 mM NaOH, 0.25% bromphenol blue, 0.25% xylene cyanol) in a final volume of 7 µl. After incubation for 3 min at 95°C, the ssDNA samples were stored on ice, loaded onto a nondenaturing polyacrylamide-like gel (0.6x MDE gel solution; Cambrex BioScience, Rockland, Maine) and electrophoretically separated at 20°C at 400 V for 18 h on a Macrophor sequencing apparatus (Pharmacia Biotech, Germany). The gel was silver stained according to the method described by Bassam et al. (2). Dried SSCP gels were digitized using an Epson Expression 1600 Pro scanner, bands with an intensity of >0.1% of the total lane were considered for further statistical analysis. Similarity coefficients were calculated using Pearson correlation algorithm. Dendrograms were constructed using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) using the GelCompare II software (Applied Maths, Kortrijk, Belgium).

5.5.7 Reamplification and sequencing of ssDNA bands from SSCP fingerprints.

Sequence information was obtained following the protocol of Eichler et al. (14). Briefly, ssDNA bands were excised from the SSCP acrylamide gels, and boiled in Tris buffer (10 mM Tris-HCl, 5 mM MgCl2, 5 mM KCl, 0.1% Triton X-100, pH 9). 7µl of the solution was used in a reamplification PCR with the unbiotinylated COM primers described above. After checking the PCR-amplicons on a 2% agarose gel, the amplicons were purified and subsequently sequenced by cycle sequencing (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit; Applied Biosystems, Foster City, Calif.). Before analysis on an ABI Prism 3100 Genetic Analyzer, the products were purified using the BigDye Terminator purification kit (QIAGEN). Phylogenetic identification of the sequences was done either by the NCBI Tool BLAST/blastn (1) for comparison with the closest 16S rRNA gene sequence or the Ribosomal Data Base Project Seqmatch Tool (10) for the identification of the closest described relative (Gene Bank Data base until September 9, 2009). To define a phylotype we chose two definite sequence differences on a mean stretch of 300bp sequence length as criterion. The

partial 16S rRNA gene sequences retrieved from the fingerprints are accessible at the GenBank/EMBL/DDBJ accession numbers GQ 917122-GQ 9171174.

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Supplementary Table 5.1: Taxonomic identification of single phylotypes of the DNA-based (1a) and the RNA based (1b) SSCP fingerprints (for phylotypes occurrence see Figure 5.3 & 5). Legend: Live/dead assigment of the phylotype: L, cells of the phylotype only present in "live", membrane intact sorted fractions; D, only present in "dead", membrane injured sorted fractions; LD, present in both fractions. N.A., not applicable (i.e., the closest described species has a similarity of < 75%).

Supplementary Table 5.1a - Phylotypes of DNA based analyses

Phylotype	Live/Dead assignment	GenBank accession no.	Taxonomic group	Closest 16S rRNA gene sequence (Accession no.)	source of closest sequence	% Similarity	Closest described species (Accession no.)	% Similarity
4	LD	GQ917124	*Betaproteobacteria*	Uncultured beta proteobacterium clone A23YP01RM (FJ569567.1)	soil, snow melt site	100	*Ralstonia syzygii* T (U28237)	100
35	LD	GQ917152	*Bacteriodetes*	Uncultured bacterium clone Lc2yS22_ML_205 (FJ355014.1)	lake Charles	99	*Sediminibacterium salmoneum* strain NJ-44 (EF407879.1)	98
37	LD	GQ917153	*Bacteriodetes*	Uncultured Bacteroidetes bacterium from DGGE gel band S1 (AY184382.1)	lake Stor Sandsjon	100	*Sediminibacterium ginsengisoli* strain DCY13 (EF067860.1)	94
39	D	GQ917154	*Bacteriodetes*	Uncultured Pedobacter sp. clone RUGL1-94 (GQ421069.1)	soil	93	*Pedobacter composti* (AB267720.1)	93
40	D	GQ917155	*Bacteriodetes*	Uncultured bacterium clone nbw601b12c1 (GQ115765.1)	skin	99	*Cloacibacterium normanense* T (AJ575430)	99
41	L	GQ917156	*Bacteriodetes*	Uncultured Bacteroidetes bacterium DGGE gel band FD 15	Baltic Sea water	99	*Polaribacter glomeratus* strain KOPRI_22229 (EU000227.1)	93

				(DQ385020.1)				
42	L	GQ917157	*Bacteriodetes*	Uncultured Bacteroidetes bacterium clone OU-3-1-1-L (EU626662.1)	sea urchin	98	*Lutibacter litoralis* T (AY962293)	98
43	L	GQ917158	*Bacteriodetes*	Uncultured Bacteroidetes bacterium clone NUD-17-1-1 (EU626712.1)	sea urchin	97	*Tenacibaculum mesophilum* (AB032504.1)	86
44	LD	GQ917159	*Alphaproteobacteria*	Uncultured bacterium clone LC10_L05A11 (FJ546770.1)	lake Cadagno	99	*candidatus Pelagibacter ubique/ Wolbachia pipientis* (AJ548800)	83
45	L	GQ917160	*Planctomycetes*	Uncultured bacterium clone FFCH623 (EU135171.1)	soil	93	*Gemmata obscuriglobus* (X85248)	87
46	LD	GQ917161	*Cyanobacteria*	Uncultured Synechococcus sp. clone XZNMC83 (EU703265.1)	oligosaline lake	100	*Synechococcus rubescens* SAG 3.81 (AM709629.1)	98
47	LD	GQ917162	*Cyanobacteria*	Uncultured cyanobacterium from DGGE band ESBAC-4 (AM261464.1)	lake Estanya	88	*Synechococcus rubescens* SAG 3.81 (AM709629.1)	86
48	LD	GQ917163	*Actinobacteria*	Uncultured bacterium clone metagen16S_cs_97 (FJ447619.1)	lake Bourget	99	*Iamibacter majanohamensis* (AB360448)	87
49	LD	GQ917164	*Actinobacteria*	Uncultured bacterium clone YU201C01 (FJ694627.1)	Yukon river	100	*Demequina aestuarii* (DQ010160)	91
50	L	GQ917165	*Gammaproteobacteria*	Uncultured bacterium clone FFCH895 (EU134767.1)	soil	93	*Methylobacter alcaliphilus* (EF495157)	87
51	D	GQ917166	*Gammaproteobacteria*	*Stenotrophomonas acidaminiphila* strain ST32 (FJ982935.1)	waste water sludge	100	*Stenotrophomonas acidaminiphila* strain ST32 (FJ982935.1)	100

52	LD	GQ917167	*Betaproteobacteria*	Uncultured bacterium clone 081127-Aspo-Fracture-Biofilm-KA1362A06 (GQ240219.1)	groundwater biofilm	100	*Acidovorax facilis* strain 228 (EU730927.1)	99
54	LD	GQ917168	*Betaproteobacteria*	Uncultured *Bordetella sp*. clone F3feb.47 (GQ417631.1)	biological degreasing system	87	*Kerstersia gyiorum* strain LMG 5906 (NR_025669.1)	87
55	L	GQ917169	*Betaproteobacteria*	Uncultured bacterium clone 3C003283 (EU801904.1)	Chesapeake Bay	84	*Polynucleobacter necessarius* (FN429668.1)	84
56	L	GQ917170	*Betaproteobacteria*	Uncultured bacterium clone LC10_L05C06 (FJ546788.1)	lake Cadagno	98	*Polynucleobacter necessarius* (FN429668.1)	98
57	L	GQ917171	*Betaproteobacteria*	Uncultured Burkholderiaceae bacterium clone LW18m-2-18 (EU642357.1)	lake Michigan	96	*Polynucleobacter necessarius subsp. asymbioticus* (FN429668.1)	86
58	LD	GQ917172	*Betaproteobacteria*	Uncultured beta proteobacterium clone LW18m-1-70 (EU642286.1)	lake Michigan	99	*Methylophilus methylotrophus* (GQ175365)	95
61	D	GQ917173	*Betaproteobacteria*	*Polynucleobacter necessarius* strain*:* USHIF010 (AB470464.1)	lake Ushikunuma	99	*Polynucleobacter necessarius* (FN429657)	99
62	L	GQ917174	*Planctomycetes*	Uncultured sludge bacterium A12 (AF234727)	wastewater sludge	94	*Zavarzinella formosa* T (AM162406)	87

Supplementary Table 5.1b (Phylotypes of RNA based analyses)

Phylotype	**Live/Dead assign-ment**	**GenBank accession no.**	**Taxonomic group**	**Closest 16S rRNA gene sequence (Accession no.)**	**source of closest sequence**	**% Similarity**	**Closest described species (Accession no.)**	**% Similarity**

1	LD	GQ917122	*Betaproteobacteria*	Uncultured bacterium clone 1C227656 (EU799977.1)	Newport harbour	100	*Acidovorax facilis* strain TSWCSN46 (GQ284412.1)	99
2	LD	GQ917123	*Betaproteobacteria*	Uncultured beta proteobacterium clone 500M5_F3 (DQ514229.1)	deglaciated soil	87	*Thauera terpenica* strain 21Mol (AJ005818.1)	87
4	LD	GQ917124	*Betaproteobacteria*	Uncultured *Ralstonia* sp. from DGGE gel band C4 (GQ255450.1)	shellfish hemolymph	100	*Ralstonia insidiosa* (FJ772078)	100
5	LD	GQ917125	*Betaproteobacteria*	Uncultured anaerobic bacterium clone C-147 (DQ018816.1)	anaerobic swine lagoon	83	*Thauera mechernichensis* (Y17590)	83
6	L	GQ917126	*Gammaproteobacteria*	freshwater Bacterium A2(2009) (GQ398339.1)	river biofilm	98	*Moraxella osloensis* strain FR1_63 (EU373514.1)	98
7	L	GQ917127	*Gammaproteobacteria*	Uncultured bacterium clone 2B20 (EU835445.1)	Reverse osmosis membrane biofilm	98	*Legionella erythra* T (Z32638)	96
8	L	GQ917128	*Gammaproteobacteria*	Uncultured bacterium clone 1B17 (EU835422.1)	Reverse osmosis membrane biofilm	80	*Legionella erythra* T (Z32638)	81
9	D	GQ917129	*Gammaproteobacteria*	Uncultured bacterium clone YSK16S-15 (EF612978.1)	acid mine drainage	91	*Legionella pneumophila; Alcoy 2300/99* (EU054324)	88
10	D	GQ917130	*Gammaproteobacteria*	*Pseudomonas koreensis* strain JDM-2 (GQ368179.1)	farm soil	99	*Pseudomonas koreensis* strain JDM-2 (GQ368179.1)	99
11	D	GQ917131	*Gammaproteobacteria*	Uncultured bacterium clone nbw232g03c1 (GQ069759.1)	skin	98	*Pseudomonas putida* strain GNL8 (FJ768454.1)	98
12	L	GQ917132	*Gammaproteobacteria*	Uncultured bacterium from SSCP band RNA 2-8-7 (DQ077602.1)	drinking water supply system	100	*Methylocaldum gracile* (U89298)	92
13	D	GQ917133	*Nitrospira*	Uncultured bacterium clone 3BR-3AA (EU937879.1)	freshwater biofilm	88	*Nitrospira moscoviensis* T (X82558)	86

14	LD	GQ917134	*Alphaproteobacteria*	Uncultured bacterium clone W_0307_65 (GQ379456.1)	soil	97	*Bosea vestrisii* T (AF288306)	97
15	LD	GQ917135	*Alphaproteobacteria*	Uncultured alpha proteobacterium clone sw-xj62 (GQ302527.1)	cold spring	97	*Pedomicrobium americanum* (X97692)	94
16	LD	GQ917136	*Alphaproteobacteria*	Uncultured alpha proteobacterium clone GASP-KB3S3_H06 (EU298674.1)	soil	99	N.A.	
17	LD	GQ917137	*Alphaproteobacteria*	Uncultured bacterium clone 0MHA_A12 (GQ306092.1)	periglacial soil	93	*Belnapia moabensis* (AJ871428)	93
18	LD	GQ917138	*Alphaproteobacteria*	Uncultured bacterium clone P1O-78 (EU375422.1)	lake Puma Yumco	98	*Roseococcus suduntuyensis* (EU012448)	96
19	LD	GQ917139	*Gammaproteobacteria*	Uncultured bacterium clone 5C231590 (EU803928.1)	lake Gatun	96	*Methylonatrum kenyense* (EU006088)	85
20	L	GQ917140	*Firmicutes*	Uncultured bacterium clone KO2_aai19h11 (EU776338.1)	Kangaroo feces	81	*Ruminococcus flavefaciens* strain AR72 (AF104841.1)	81
21	LD	GQ917141	*Planctomycetes*	Uncultured planctomycete, clone DSP41 (AJ290189.1)	river Spittelwasser biofilm	94	*Rhodopirellula baltica* (FJ624344)	85
22	L	GQ917142	*Bacteriodetes*	Uncultured bacterium clone HH1409 (FJ502249.1)	lake Cadagno	98	*Pedobacter sp.* Tianshan 221-3 (EU305635.1)	93
23	D	GQ917143	*Bacteriodetes*	Uncultured Bacteroidetes bacterium clone A21YG08RM (FJ568900.1)	soil at snow melt site	95	*Flexibacter canadensis* (AB078046)	89
24	LD	GQ917144	*Chloroflexi*	Uncultured bacterium clone 538.F4 (EU357588.1)	soil	99	N.A.	
25	LD	GQ917145	*Chloroflexi*	Uncultured bacterium clone: CMBR-4 (AB305032.1)	wastewater treatment plant	91	*Caldilinea aerophila* (AB067647)	83

26	L	GQ917146	*Cyanobacteria*	Uncultured bacterium from SSCP band Li-8R-10-2 (DQ077567.1)	drinking water supply system	95	*Glaucocystis nostochinearum* (X82496)	79
27	L	GQ917147	*Cyanobacteria*	Uncultured bacterium from SSCP band TW15-RNA1-14-2 (DQ077556.1)	drinking water supply system	94	*Glaucocystis wittrockiana* (X82495)	83
28	LD	GQ917148	*Bacteriodetes*	Uncultured bacterium clone F126 (FJ348594.1)	waste water sludge	99	*Thermolithobacter carboxydivorans* (DQ095862)	90
29	D	GQ917149	*Cyanobacteria*	Uncultured bacterium clone IFBC1H11 (EU592534.1)	freshwater lake	88	*Synechococcus sp. KORDI-78* (FJ497748.1)	87
31	LD	GQ917150	*Cyanobacteria*	Uncultured bacterium clone N05Dec-74 (EU442941.1)	lake Nam Co	90	*Cyanobium sp.* JJM10A4 (AM710358.1)	90
32	L	GQ917151	*Cyanobacteria*	Uncultured bacterium clone LaP15L91 (EF667687.1)	river sediment	97	*Synechococcus sp.* MH305 (AY224198.1)	100
46	LD	GQ917161	*Cyanobacteria*	Uncultured Synechococcus sp. clone XZNMC83 (EU703265.1)	lake Namucuo	100	*Synechococcus rubescens* SAG 3.81 (AM709629.1)	98

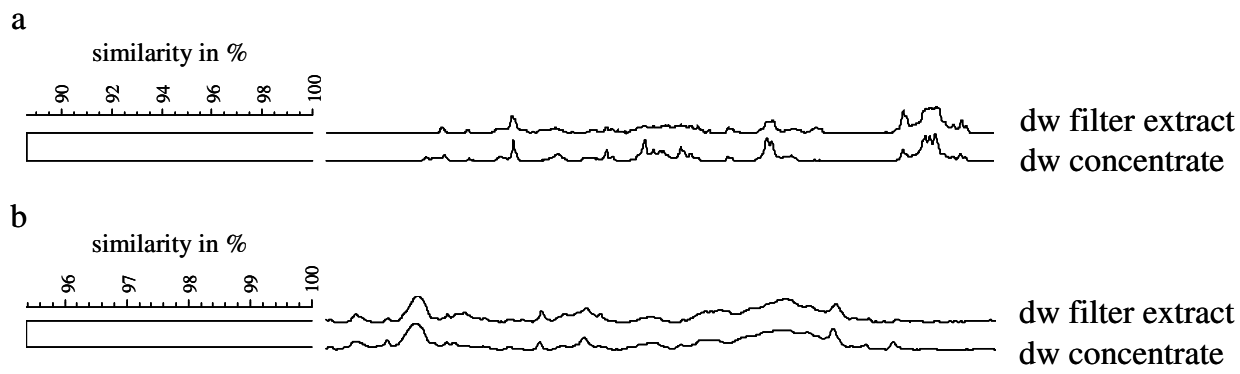
*Supplementary Table 5.2: Abundances of phylotypes sorted according to the respective Phyla/class. The abundances of the phylotypes are derived from the SSCP analyses of DNA (Table 2a) and RNA (Table 2b) extracts as shown in Figure 5.4. The mean of the three samplings A,B and C plus the standard deviation SD is given for the tap water sample before sorting ("All (unsorted)"), the "live" sorted fraction (cells with intact membranes), and the "dead" sorted fraction (cells with injured membranes). "Sum" indicates the sum of the abundance of all phylotypes of the same phyla/class, "SD" shows to the standard deviation of the sum per sampling day (A-C). Legend: PT, phylotype; phylotype signature corresponds to Supplementary Table 1.; n.d., not detected; *, phylotypes retrieved from both RNA and DNA extracts*

Supplementary Table 5.2a (DNA based analyses)

DNA based analyses		**All (unsorted)**		**Live**		**Dead**	
Phyla/Class	**PT signature**	**mean A-C**	SD	**mean A-C**	SD	**mean A-C**	SD
Alphaproteobacteria	44	**2.83%**	2.24%	**1.56%**	1.48%	**0.92%**	1.49%
Betaproteobacteria	52	**2.71%**	1.28%	**1.97%**	3.12%	**0.59%**	1.03%
	4*	**0.25%**	0.22%	**8.11%**	8.43%	**4.96%**	8.60%
	54	**0.20%**	0.30%	**0.18%**	0.26%	**1.83%**	2.56%
	55	**0.78%**	1.35%	**1.29%**	1.27%	**n.d.**	
	56	**4.81%**	2.31%	**1.78%**	2.27%	**n.d.**	
	57	**1.20%**	0.87%	**2.06%**	1.51%	**n.d.**	
	58	**4.56%**	0.10%	**1.42%**	1.23%	**1.19%**	2.06%
	61	**0.42%**	0.72%	**n.d.**		**1.35%**	2.34%
	Sum	**14.93%**	0.52%	**16.82%**	6.55%	**9.92%**	10.80%
Gammaproteobacteria	50	**0.34%**	0.59%	**0.50%**	0.86%	**n.d.**	
	51	**n.d.**		**n.d.**		**1.05%**	1.81%
	Sum	**0.34%**	0.59%	**0.50%**	0.86%	**1.05%**	1.81%
Actinobacteria	48	**6.87%**	3.85%	**2.35%**	3.19%	**2.01%**	3.48%
	49	**8.45%**	3.86%	**2.17%**	0.52%	**3.67%**	3.24%
	Sum	**15.32%**	1.12%	**4.52%**	3.71%	**5.68%**	6.11%
Bacteroidetes	35	**12.38%**	0.28%	**2.46%**	1.80%	**7.14%**	3.74%
	37	**1.34%**	1.18%	**0.57%**	0.64%	**0.97%**	1.26%
	39	**0.08%**	0.13%	**n.d.**		**0.23%**	0.40%
	40	**1.66%**	0.45%	**n.d.**		**0.15%**	0.26%
	41	**2.23%**	3.87%	**4.79%**	8.30%	**n.d.**	
	42	**n.d.**		**5.98%**	10.35%	**n.d.**	
	43	**0.06%**	0.10%	**1.65%**	2.86%	**n.d.**	
	Sum	**17.75%**	4.56%	**15.45%**	22.67%	**8.49%**	3.02%
Cyanobacteria	46*	**1.31%**	1.24%	**n.d.**		**9.50%**	16.46%
	47	**1.99%**	0.29%	**0.64%**	1.10%	**0.60%**	1.03%
	Sum	**3.30%**	0.97%	**0.64%**	1.10%	**10.10%**	17.49%
Planctomycetes	62	**n.d.**		**10.80%**	18.71%	**n.d.**	
	45	**0.22%**	0.38%	**2.01%**	3.48%	**n.d.**	
	Sum	**0.22%**	0.38%	**12.81%**	22.19%	**n.d.**	
% identified as PTs		**54.69%**	3.83%	**52.29%**	21.52%	**36.16%**	23.66%

Supplementary Table 5.2b (RNA based analyses)

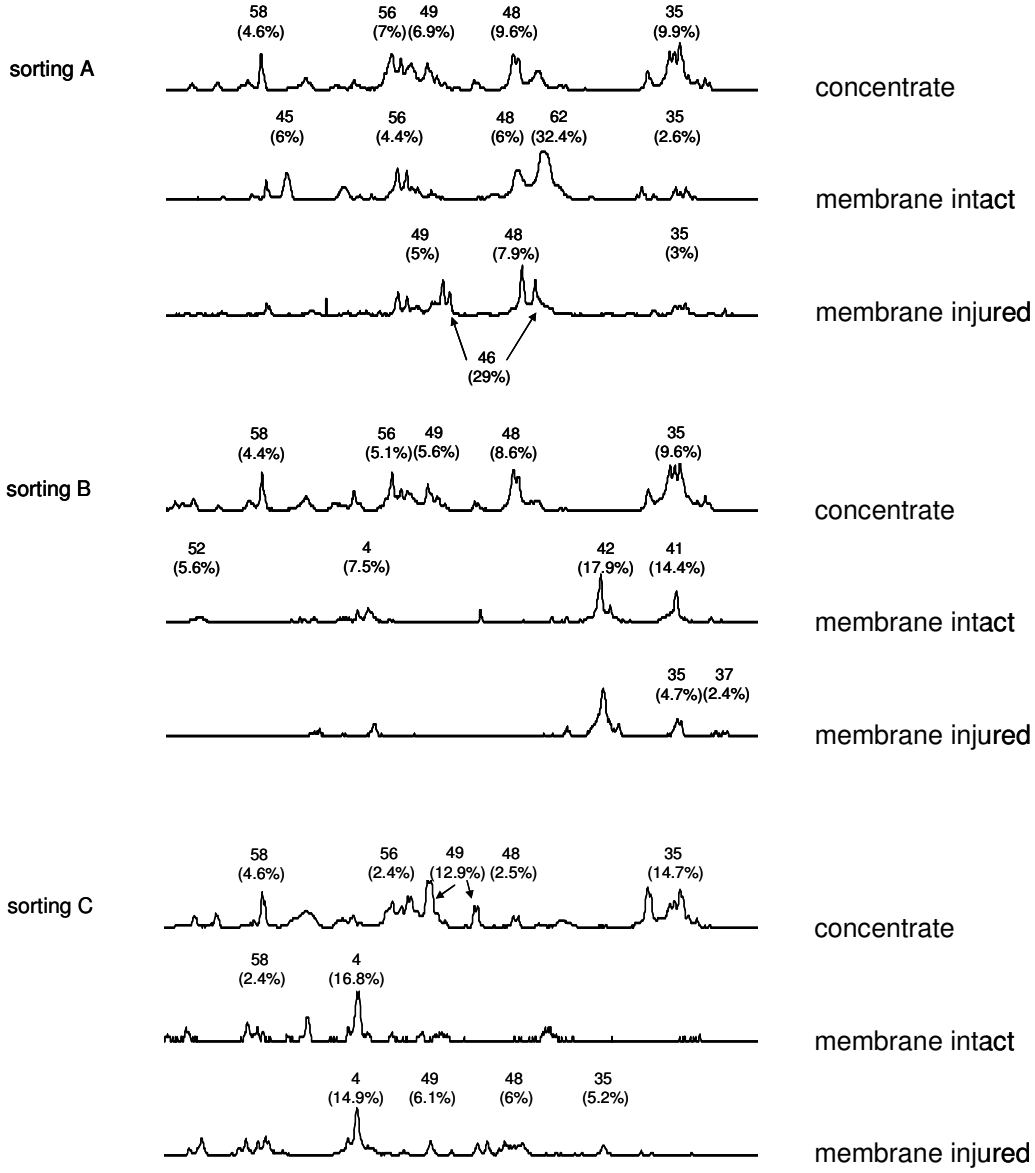
RNA based analyses		All (unsorted)		Live		Dead	
Phyla/ Class	**PT signature**	**mean A-C**	SD	**mean A-C**	SD	**mean A-C**	SD
Alphaproteobacteria	14	**13.03%**	7.38%	**5.05%**	1.85%	**7.50%**	5.32%
	15	**0.49%**	0.85%	**0.74%**	1.29%	**1.99%**	1.92%
	16	**1.21%**	2.09%	**7.12%**	9.13%	**0.09%**	0.15%
	17	**n.d.**		**4.38%**	7.59%	**0.92%**	1.60%
	18	**0.76%**	1.31%	**3.75%**	3.64%	**1.02%**	1.76%
	Sum	**15.49%**	11.41%	**21.04%**	20.67%	**11.52%**	8.19%
Betaproteobacteria	1	**18.14%**	5.22%	**5.22%**	1.88%	**5.89%**	1.39%
	2	**0.90%**	1.01%	**0.49%**	0.49%	**0.59%**	1.03%
	4*	**0.81%**	1.26%	**3.57%**	2.81%	**3.85%**	2.48%
	5	**0.91%**	0.79%	**n.d.**		**n.d.**	
	Sum	**20.76%**	5.66%	**9.28%**	3.14%	**10.33%**	4.27%
Gammaproteobacteria	6	**1.72%**	2.98%	**3.14%**	1.61%	**n.d.**	
	7	**0.14%**	0.24%	**1.84%**	1.33%	**n.d.**	
	8	**n.d.**		**1.86%**	3.22%	**n.d.**	
	9	**0.53%**	0.92%	**n.d.**		**3.01%**	2.67%
	10	**0.02%**	0.03%	**n.d.**		**2.36%**	1.50%
	11	**n.d.**		**n.d.**		**7.53%**	8.81%
	12	**1.38%**	0.17%	**9.10%**	1.01%	**n.d.**	
	19	**5.72%**	1.01%	**1.64%**	1.42%	**1.38%**	1.39%
	Sum	**9.50%**	4.02%	**17.58%**	5.60%	**14.27%**	11.31%
Bacteroidetes	22	**1.50%**	2.60%	**1.29%**	1.32%	**n.d.**	
	23	**6.63%**	1.62%	**n.d.**		**1.80%**	3.12%
	28	**0.18%**	0.31%	**1.20%**	1.26%	**0.69%**	0.86%
	Sum	**8.31%**	2.64%	**2.49%**	2.31%	**2.49%**	3.96%
Chloroflexi	24	**0.70%**	0.47%	**13.67%**	8.14%	**1.67%**	1.78%
	25	**n.d.**		**2.08%**	1.38%	**1.62%**	1.98%
	Sum	**0.70%**	0.47%	**15.75%**	9.10%	**3.28%**	2.13%
Cyanobacteria	26	**2.93%**	0.47%	**4.42%**	7.66%	**n.d.**	
	27	**n.d.**		**0.98%**	0.68%	**n.d.**	
	29	**0.48%**	0.83%	**n.d.**		**1.51%**	2.61%
	46*	**10.48%**	7.38%	**2.20%**	1.72%	**14.72%**	5.59%
	31	**0.88%**	0.83%	**n.d.**		**n.d.**	
	32	**0.82%**	1.41%	**0.25%**	0.44%	**n.d.**	
	Sum	**15.59%**	7.56%	**7.85%**	7.33%	**16.23%**	8.13%
Firmicutes	20	**2.25%**	1.95%	**0.95%**	1.64%	**n.d.**	
Nitrospira	13	**0.10%**	0.17%	**n.d.**		**1.82%**	1.84%
Planctomycetes	21	**4.60%**	7.96%	**n.d.**		**n.d.**	
% identified as PTs		**77.30%**	3.39%	**74.93%**	20.17%	**59.94%**	5.68%



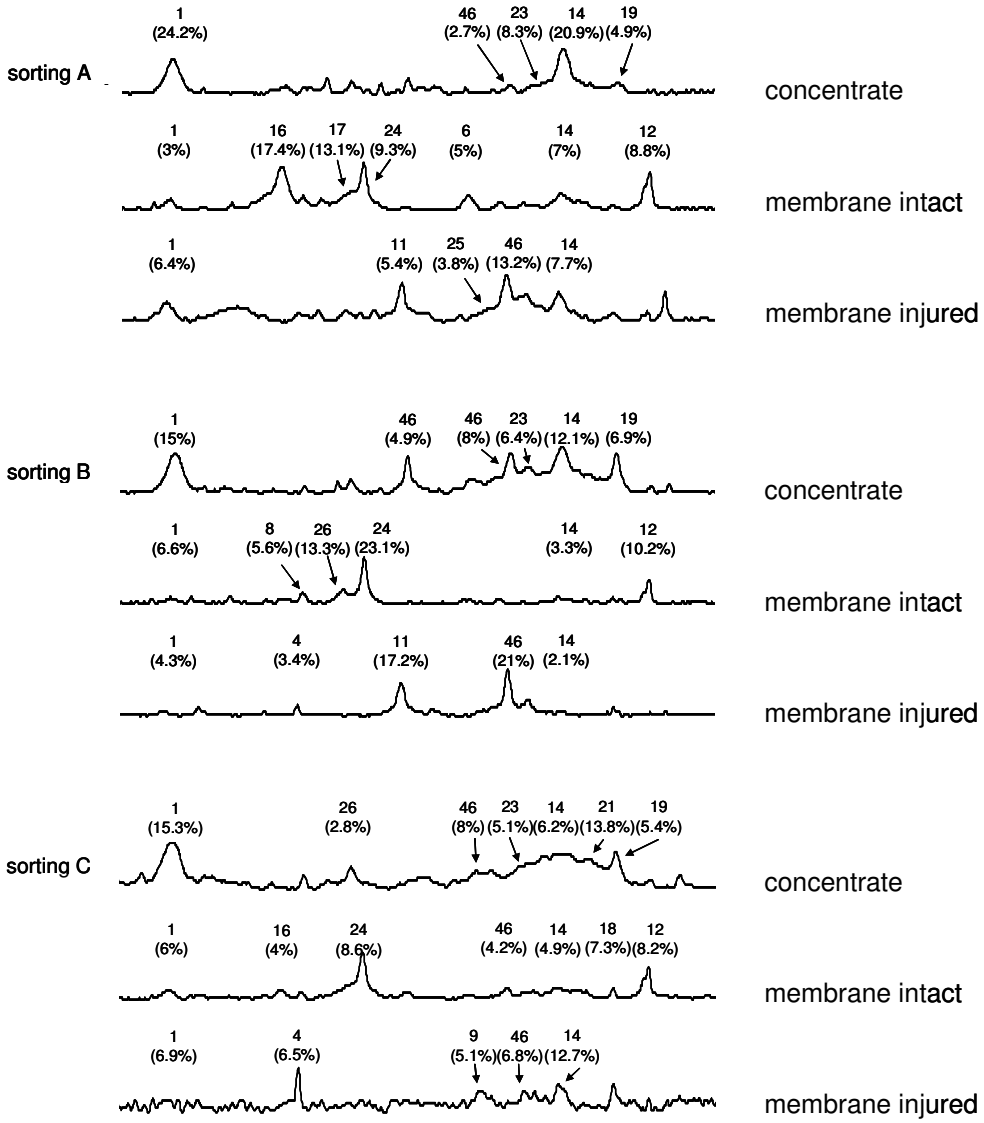
Supplementary Figure 5.1: Comparison of SSCP electropherograms of concentrated drinking water samples directly extracted from concentrates and non-concentrated drinking water extracted from filter sandwiches using GelCompare II. (a) DNA-based SSCP electropherograms (b) RNA-based SSCP electropherograms.

Supplementary Figure 5.2

a) DNA based SSCP



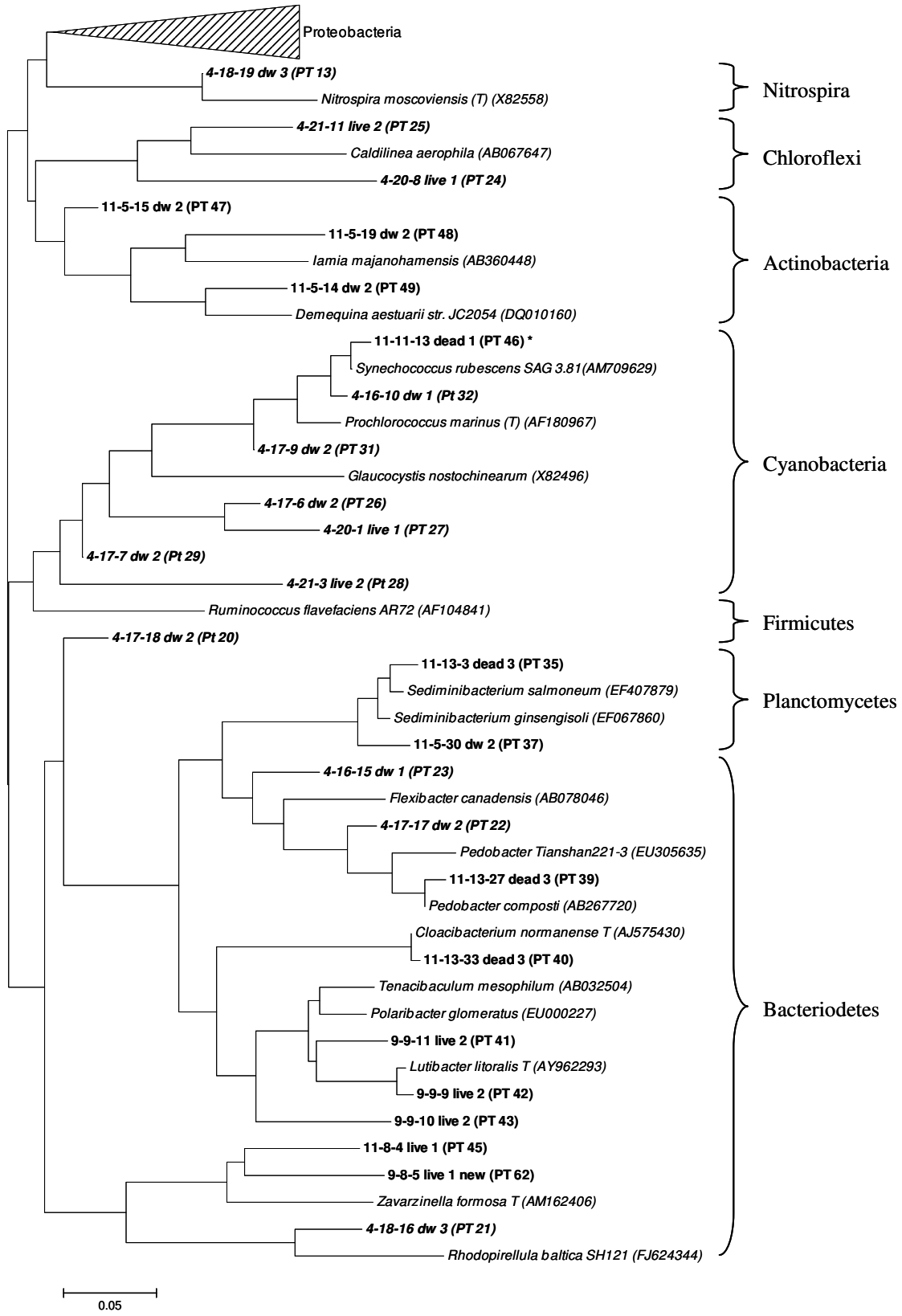
b) RNA based SSCP



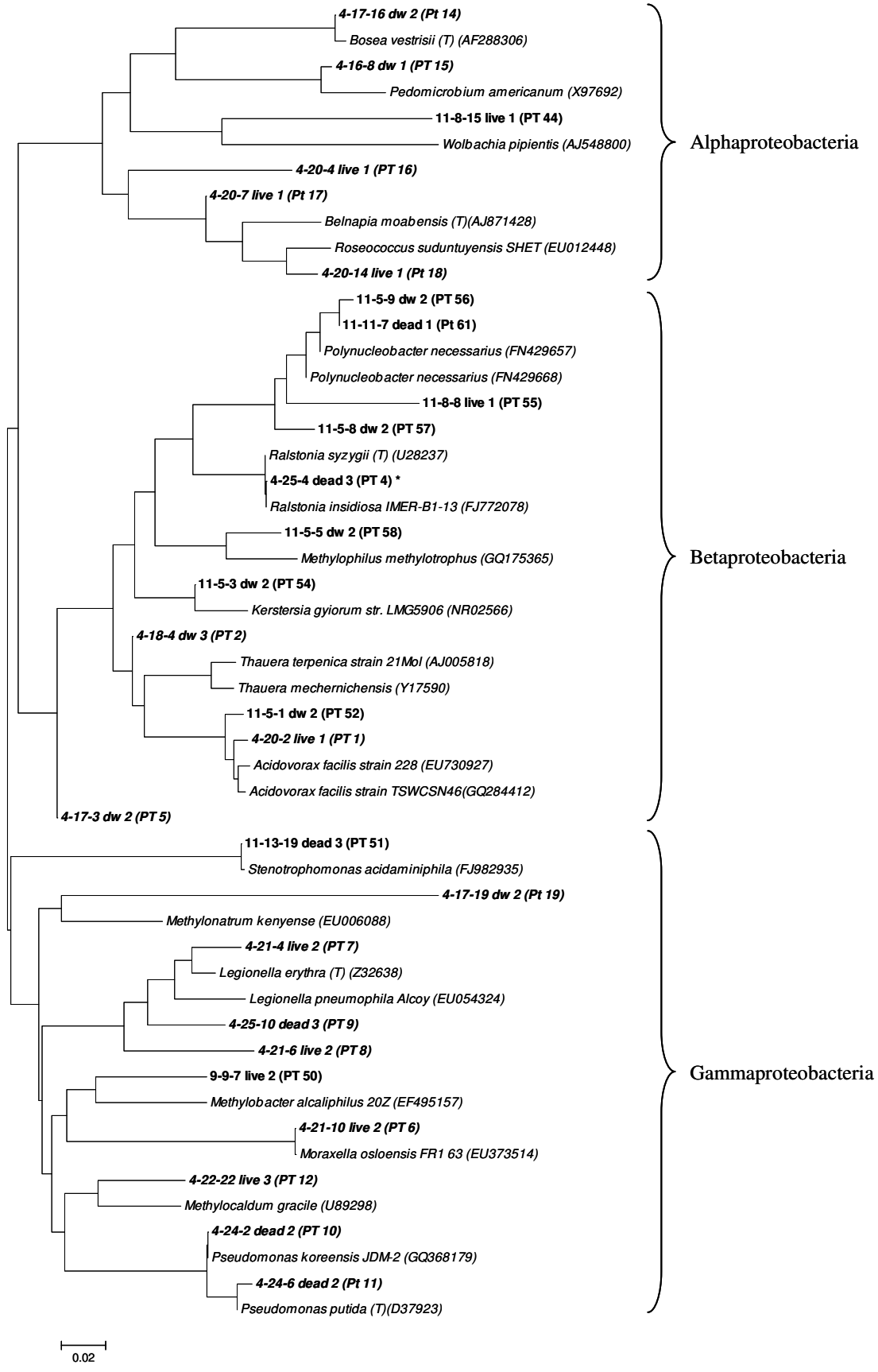
Supplementary Figure 5.2: (a) Detailed analysis of the electropherograms from the different sampling dates originating from the DNA-based SSCP gel given in Figure 5.4a. Numbers correspond to the phylotypes given in Tab 1. Percentages in parentheses represent relative abundances of phylotypes. (b) Detailed analysis of the electropherograms from the different sampling dates originating from the RNA-based SSCP gel given in Figure 5.4b.

Supplementary Figure 5.3

a) all phylotypes



b) Phylotypes affiliated with Proteobacteria



Supplementary Figure 5.3: Phylogenetic analysis of 16S rRNA gene sequences obtained from the bands of the SSCP fingerprints shown in Figure 5.4 using the neighbor-Joining method. The optimal tree with the sum of branch length = 2.1515 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pair wise sequence comparisons (pair wise deletion option). Sequences are labeled with their origin plus the phylotype number (in parenthesizes) given in supplementary Table 1 and 2. Sequences are coded with different character types according to their origin in terms of nucleic acid type: DNA-based sequences are shown in bold, RNA-based sequences are shown in bold italic, and sequences occurring in DNA- and RNA-based fingerprints are shown in bold with an asterisk. (a) Phylogenetic tree of all detected phylotypes. (b) Phylogenetic tree of the detected phylotypes affiliated with the Proteobacteria.

6 General Discussion

6.1 Influence of the environment on the seasonality of the cold drinking water community

Monthly sampling was performed with the drinking water of a drinking water supply system (DWSS) located in Northern Germany and stemming from two reservoirs in the Harz Mountains (Chapter 1.3). Starting in September 2006 and ending in October 2009 drinking water from the tap on the HZI was sampled at monthly intervals. Starting in May 2008, also relevant drinking water parameters, such as pH, conductivity, temperature, and chlorine concentration were determined. With these samples, two studies were performed.

In the first study (Chapter 2), we applied DNA based SSCP fingerprinting of the samples from September 2006 to April 2008 to get an overview of the seasonal dynamics of the community structure. It was shown, that the seasonal dynamics were characterised by 40-80 bands of varying intensities and at least 3 major bands that were constantly present over the whole sampling period (Fig 2.1). Excising and sequencing of the constant bands resulted in three phylotypes. One phylotype belonged to the phylum *Actinobacteria*, and two phylotypes belonged to the class *Alphaproteobacteria* and *Betaproteobacteria*. These three phylotypes have already been detected in a former study of the same DWSS (7). The varying bands in the SSCP-gel occurred during specific periods and could therefore be seen as indicators of changes in the community structure. This confirmed in general, that there was a seasonal variation in the microbial drinking water community. As Eichler et al. showed in their previous study that the taxonomic composition of the drinking water community reflects the composition of both drinking water reservoirs in the Harz Mountains, our study showed that both source waters had a significant influence in their seasonality on the composition of the source water.

To determine in detail the variation of the community structure and composition and to identify factors influencing the changes in the community, a second study was performed. In the second study, SSCP fingerprints were applied for the samples from May 2008 to October 2009. In addition, total bacterial counts were performed and relevant drinking water parameters such as pH or temperature were determined. Fingerprints based on DNA were used to determine the present community

composition, while RNA based fingerprints were used to assess the activity of the community. The analyses included SSCP fingerprinting, sequencing of most bands and the phylogenetic assignment of the 16S rRNA fragments. Relative abundances were determined and put into relation to meteorological data.

A strong negative correlation between the change in total bacterial counts of the drinking water and the precipitation regime was observed, showing a dilution effect (Fig 3.1). Thus, we assume an inflow of water with relatively low nutrient content into the reservoirs leading to a dilution effect. The precipitation in the catchment area was the major impact influencing the amount of bacteria in cold drinking water. According to Niquette et al. who reported a correlation between high drinking water temperature and bacterial activity and growth, the second important environmental factor influencing the concentration of bacteria we observed was the drinking water temperature (30).

In line with the first study on seasonal variation, the clustering of the fingerprints revealed reoccurring similarities in seasonal drinking water community structures and community activities. Not only similarities of both types of fingerprints between different seasons were found, compared with former studies of the drinking water of the Harzwasserwerke the fingerprints bear a high resemblance to those fingerprints (7, 10, 16). Interestingly, the phase transitions from one subcluster to another in the dendrogram of the RNA based fingerprints were corresponding with strong decreases in total bacterial numbers and month of strong precipitation while the transition phases in the DNA based dendrogram did not show any correlation to precipitation (Fig 3.2 a and b). A month of high precipitation and thereby changed environmental parameters had presumably a strong impact on the activity of bacteria in cold drinking water, while the community structure itself was not affected by precipitation regime.

In the seasonal dynamics plot of the DNA based fingerprints (Fig 3.5 a), two main phases of the present bacterial community were observed, a summer and a winter phase. Transitions from one seasonal community to the other coincided with times of mixing events in dimictic lakes in spring and autumn. It can be inferred that the bacterial community changes in cold drinking water reflected the community changes in the dimictic reservoirs. For the RNA based fingerprints, phases of high activity of single phylotypes were temporally limited and clearly separated, for instance a phase of high activity of *Cyanobacteria* in winter 2008/2009. The coincidence of pronounced decreases in the change of total bacterial numbers with every phase transition and the

coincidence of months of high precipitation with almost every phase transition could be a hint of a causal relationship. It is conceivable that months with high precipitation or other environmental impacts like the mixing of the reservoir created different environmental conditions leading to a collapse of the existing bacterial community, followed by a regrowth of the community to higher levels until the next month of heavy rainfall. Interestingly, these phases of high activity were only observed in single phylotypes and not in all members of the taxonomic group. This could mean that these phylotypes were highly specialized to the respective environmental conditions. It is also conceivable, that some phylotypes posess a high 16S-rRNA content due to other reasons. The high level of cyanobacterial rRNA in the winter might be explained by a storage function of RNA for phosphorus and nitrogen in *Cyanobacteria* (1, 5, 21). In addition, some taxonomic groups tended to possess low rRNA-levels despite their high abundance on DNA-based fingerprints such as. *Actinobacteria* and *Bacteroidetes*, both a small sized, which is thought to be reason for their low ribosome content (9, 29). In general, there was a great discrepancy between DNA based and RNA based fingerprints with only 7 out of 43 phylotypes overlap. Thus, it can be inferred that many active bacterial phylotypes were not detectable in DNA based fingerprints.

6.2 Hot drinking water communities

Corresponding to the cold drinking water sampling, hot drinking water was sampled during 1.5 years. It was sampled in May 2008, and regular monthly sampling was done from September 2008 to October 2009. Hot water was taken from a shower with several minutes flushing to avoid the sampling of stagnated water. The hot drinking water was made from normal cold drinking water directly on the campus where it was heated to 60°C and transferred in insulated pipes to the shower using a circular supply system. Similar to the cold drinking water study, total bacterial counts were performed. Fingerprints based on DNA were used to determine the present community composition, while RNA based fingerprints were used to assess the activity of the community. The analyses included SSCP fingerprinting, sequencing of all relevant bands and the phylogenetic assignment of the 16S rRNA fragments.

The number of bacteria measured in the hot drinking water was only 20% lower than in cold, unheated drinking water, although the heating of drinking water to temperatures of 60°C is widely used to reduce total bacterial numbers and inactivate a

number of pathogens such as *Legionella* (35). A conceivable hypothesis for these unexpected high cell numbers could be an initial decay of the bacterial microflora due to heating and following a regrowth of those bacteria that are not susceptible to high temperatures. Nutrient limitation is a major cause of limiting growth in drinking water (27). When cold drinking water is heated to hot drinking water, the killing of heat susceptible cells may lead to a release of organic and inorganic nutrients. The released nutrients may allow regrowth to comparable abundances bacteria had before. This would explain the similar dynamics of the hot water bacterial counts compared to the cold water bacterial counts until June 2009 despite their different community structure.

The seasonal variability of the bacteria in hot drinking water was rather low, confirmed for both, DNA and RNA based fingerprints. Nevertheless, for both kinds of fingerprints, fingerprints of the same season clustered together, indicating a weak temporal variation maybe influenced by parameters such as nutrient concentration or varying usage of hot water due to different seasons.

It was shown before for domestic hot water that the ratio between HPC and AODC in hot water was approximately 20 times higher than the ratio in cold drinking water (2). Therefore, it became clear that there were substantial differences in both community structures. The mean richness of cold drinking water was markedly higher than the richness of hot water. In addition, in hot water the mean first rank abundance was clearly higher and the slope of the exponential regression in a logarithmic plot was clearly steeper than these measures in cold drinking water. Only few species may be adapted to an environment with extreme parameters like in hot water causing a low richness (4). These adapted species may grow in this environment with reduced competition and grazing by protozoa leading to higher abundances than in cold drinking water. Possibly, as many species compete in cold drinking water for the same resources the relative abundances were lower than those in hot drinking water. Generally these differences in diversity measures were strong indicators for a markedly different bacterial community structure in cold and hot drinking water.

In both, DNA and RNA based fingerprints, we found the same core community represented by the same five phylotypes, each belonging to following taxonomic groups: *Betaproteobacteria*, *Acidobacteria*, *Alphaproteobacteria*, *Bacteroidetes* and *Planctomycetes*.

In contrast to cold water, present bacteria detected by DNA based fingerprints were also active, as the same phylotypes were found in the active community. This effect

could be explained by regrowth: the cold water community was mostly destroyed by heating of the water up to 60°C, and subsequently only those bacteria adapted to high temperatures were growing to recolonize the hot drinking water, i.e. the thermophilic and thermotolerant bacteria. Two effects may influence the building of such a community: 1) Selection of thermophilic bacteria from the seedbank and 2) selection of thermotolerant phylotypes from the core community of the cold drinking water.

Most of the phylotypes found in hot water were assigned to be of “hot habitat origin”, although the source of the hot drinking water did not have any contact to hot habitats. These phylotypes were considered to be thermophilic. This distance to any hot habitats supports the model of "abundant and rare members" describing the bacterial community composition in pelagic environments. In this model, the community consists of a core community with few taxa that are highly abundant and a huge seed bank with nearly infinite numbers of very low abundant phylotypes (13, 14, 32). As the hot drinking water provides niches that differ from those in the cold drinking water, we hypothesize that the low abundant thermophilic bacteria from the cold drinking water seed bank were recruited forming partly the core community in hot drinking water. The second most prevalent group consisted of phylotypes already found in the cold drinking water of the Harzwasserwerke. Thus, a selection for those thermotolerant bacteria of the cold drinking water community happened, that were able to survive water temperatures of 60°C.

Because the core community in DNA based and RNA based fingerprints was very similar in hot drinking water, we conclude that in hot drinking water a high amount of 16S rRNA is an indicator for activity and growth. However, in cold drinking water the situation was different. The high abundant bacteria were not necessarily the actives ones, while the active bacteria were not necessarily of high abundance. We assume that the activity of cold drinking water bacteria is dependent on different factors than the activity of hot water bacteria. While the dynamics of the rRNA abundance in cold drinking water is strongly influenced by environmental parameters such as precipitation and temperature, the dynamics of the hot drinking water were not or only on a very low level influenced by these parameters. Although it can be assumed that the organic and inorganic nutrient levels in hot water had not considerably changed during the heating process, the dynamics of the activity of the hot drinking water community seemed to be less dependent on changes in environmental conditions than the dynamics of the activity of the cold drinking water community.

6.3 Bulk water and drinking water biofilms

To investigate the community composition of mature drinking water biofilms together with the drinking water, a sampling of both was performed. Bulk water was sampled from several taps distributed on the campus of the Helmholtz Centre for Infection Research (HZI) and from two households of the inner city of Braunschweig. Biofilm samples were taken in parallel to the bulk water sampling. Additional biofilm samples were obtained when a building at the HZI campus was dismantled. All bulk water or biofilm samples were sampled in three different water networks connected to the HZI campus, the Municipal water network, the main network at the HZI campus and the looped fire water mains. All samples were used to perform 16S rRNA SSCP fingerprinting from DNA and RNA. To investigate similarities between the community structure of the samples, cluster analysis of the fingerprints was done. Major bands of the fingerprints were excised and sequenced in order to perform a phylogenetic assignment of the 16S rRNA sequences.

Consistent with Eichler et al. (7), who showed, that after chlorination, the drinking water community was almost identical all along the DWSS, we found a high similarity of all bulk water fingerprints demonstrating the stability of the overall community structure of the drinking water bacteria with little spatial variation. We observed clearly separated subclusters for DNA- and RNA-based fingerprints indicating large differences between the present and the active bacteria in bulk water. In contrast to bulk water, biofilm fingerprints showed large differences of the bacterial communities present. Each biofilm showed a unique pattern of bands indicating that each biofilm consisted of a unique community. As the community structure is influenced by environmental conditions (34), each biofilm habitat seemed to have its own micro-environmental conditions like pH, oxygen concentration or nutrient availability, provided not only by surface material or water quality, but also by the community itself. These findings were confirmed by the comparative cluster analysis of all biofilm fingerprints (Figure 4.4b). In general, the clustering distance reflected the physical vicinity between the biofilms more than surface material properties. The observed similarity of physically related biofilms and the low dependency of the community structure on the surface material could be explained by the mutual influence of adjacent biofilm communities. Although the first colonisation of surfaces is dependent on the surface material (6, 18), an adjacent

coexistence for years may lead to mutual influence of biofilms by exchange of bacteria. It is conceivable, that once the surface is covered by a first, material specific biofilm, it is overgrown by a nearby biofilm community that is more independent from the surface material. From our observations, we hypothesize that during several years physically related biofilm communities will show similar community structures. Confirming our observation, Martiny et al. showed for their model DWSS that after three years most biofilms from different sampling positions clustered together and therefore possessed a homogeneous bacterial composition (25).

All bulk water communities showed higher richness or indices that are estimates of richness than biofilm communities (Table 4.3). We assume that only those bacteria were successful in colonizing biofilms that can actively contribute to the succession of the biofilm, while those bacteria that cannot fill perfectly the narrow niches in biofilms vanished after time. This process would lead to a lower richness in biofilm than in the corresponding bulk water. This assumption would also explain why lower richness values were found for biofilms than for bulk water. Biofilms investigated in our study were definitely older than three years, therefore we can assume that the trend of deminishing number of species, shown by Martiny et al., continued to reach a lower richness than the corresponding bulk water (25).

Almost all of these phylotypes obtained from bulk water fingerprints were considered to be of aquatic origin and belonged to typical freshwater taxonomic groups (28, 38). Most of the phylotypes were identical, or at least very similar to those formerly observed by Eichler et al. (7) and Kahlisch et al. (16). Although seasonal changes in the community structure were demonstrated before (Chapter 2 and 3), the overall composition of the community in the DWSS remained rather constant for about four years (10). This is evidence that the concept of a stable "core community" is applying to bulk drinking water communities from man-made freshwater environments leading to good resilience to temporally and spatially limited disturbances of the bacterial community (14). The majority of biofilm phylotypes were, in contrast to bulk water, considered to be of soil, sludge or sediment origin or of biofilm origin. Members of the key genera *Rhizobiales*, *Nitrospira* or *Thiobacillus* which we found in drinking water biofilms, are known to contribute to the biogeochemical cycling of nitrogen or sulphur. Also many other uncultured bacteria with high similarities to denitrifying species were found in these biofilms. This suggests that the species in biofilms form a system of complex interactions to build a community metabolism. Although each biofilm consists

of a set of unique phylotypes, these phylotypes belonged to classes, which were present in most biofilms in comparable abundances, especially members of the *Alphaproteobacteria*, *Gammaproteobacteria*, and *Deltaproteobacteria* (Figure 4.6 b, 4.7). This may indicate that different biofilm communities provide niches with similar conditions which are indeed filled by different species belonging to the same class (Tab. S3).

There was no overlap in the phylotypes (using a detection limit of 0.1% relative abundance) stemming from the bulk water community or the biofilm community. Thus, it can be inferred that no major exchange between the two core communities occurred. The current model of "abundant and rare members" describes the microbial community in pelagic ecosystems, consisting of a core community with few taxa that are highly abundant and a seed bank with nearly infinite numbers of very low abundant phylotypes (13, 14, 32). As a biofilm provides niches that differ from those in the bulk water, we hypothesize that the low abundant and therefore not detected bacteria from the bulk water seed bank were recruited for the biofilm development and biofilm succession. This represents a possible mechanism for transition from a rare member of the bulk water community to an abundant member of the biofilm community. (3)

6.4 Assessment of live and dead drinking water bacteria

A combined molecular and cellular approach was performed for this study. In detail SYTO9 and Propidium Iodide (PI) was used for a live/dead staining, followed by Fluorescence Activated Cell Sorting (FACS) and community fingerprinting with a subsequent 16S rRNA (gene) sequence analysis of the major bands of the fingerprints. Drinking water was stained on three sampling dates with the live/dead staining kit and subsequently subjected to FACS. The live fraction, i.e. the membrane intact fraction, and the dead fraction, i.e. the membrane damaged fraction, were separated and compared to unsorted cells from the drinking water. The three fractions, live sorted cells, dead sorted cells and unsorted cell wre then submitted to nucleic acid extraction and subsequently analysed using 16S rRNA (gene) based Single Stranded Conformation (SSCP) analysis. Major bands were excised and sequenced to provide insight into the taxonomic composition of the bacterial community of the single fractions.

The drinking water community determined in this study is consistent with the findings of former studies of the respective drinking water supply system (Chapter 2-4).

In the studied drinking water, about half of the bacterial cells showed an intact membrane. This is in line with other studies reporting a fraction of membrane intact cells of about 66% in chlorine free tap water as it was the case in the present study (3). For chlorine containing tap water, Hoefel et al. reported 12% membrane intact cells for finished drinking water of an Australian water distribution system (11). The live/dead staining procedure using the combined dyes SYTO9 and PI to distinguish between live and dead cells was shown to be good estimate for membrane injury of bacteria (22), and many studies evaluated and compared it with other methods extensively (8, 15).

After sorting into the live or dead fraction, and a subsequent SSCP analysis, all DNA-based phylotypes that were detected in the unsorted fraction were retrieved in the live or dead fraction. For the RNA based phylotypes the rate of retrieved phylotypes was 90%. This nearly complete recovery of the phylotypes after cell sorting allows a comparison of the sorting results between the DNA- and RNA-based analyses.

The fraction of membrane injured cells reflected by the DNA based analyses was not higher than those of the RNA based analyses. In other studies it was often assumed that phylotypes reflected by the RNA are alive, while those reflected by DNA are dead. Thus, we assume that the phylotype-specific regulation of the DNA and RNA pool is not related to the viability of the respective phylotype. This is consistent with analyses of Klappenbach et al. showing a broad range of numbers of rRNA operons specific for each bacterial strain, ranging from 1 to 13 (19). On the other hand, all fingerprints of the membrane intact fractions showed rather similar RNA-based fingerprints reflecting actively growing members of the community. This could indicate that always the same actively growing members of the drinking water community regrew after chlorination.

Autofluorescence has taken into account as a possible bias of the cell sorting procedure using SYTO9 and PI (37). Two phylotypes were affiliated with the phylum *Chloroflexi*, which is known to contain bacteriochlorophyll a and c, causing a green autofluorescence (23). Another phylotype was affiliated with *Synechococcus*, which is known to produce the red fluorescent phycoerythrin (36). The phylotypes affiliated with *Chlorofexi* had higher abundances in the live sorted fraction, while the phylotype affiliated with *Synechococcus* had a higher abundance in the dead sorted fraction. Thus, a wrong live/dead sorting of these three phylotypes cannot be excluded. Though autofluorescence may be misleading for the live/dead sorting of some bacteria with photosynthetic pigments, we do not consider this as a critical issue for the live/dead staining procedure as a distinction for drinking water bacteria. Autofluorescent bacteria

are commonly not considered as pathogenic and therefore, autofluorescence does not seem a critical issue for our staining procedure in terms of public health.

Overall, we think that the combination of FACS sorting and fingerprinting is a new way to obtain functional fingerprints – with the live RNA phylotypes representing the most actively growing members of the microbial community.

6.5 Outlook

Molecular fingerprinting techniques formed the backbone for analysing the bacterial microbiome in the respective community in all studies performed in this thesis. The future will bring progress into these molecular fingerprinting methods. For example, new sequencing technologies such as pyrosequencing (Chapter 1.8) will overcome several drawbacks: the readout length of the sequences and the cost-efficiency of a single run will increase (24, 26). By this, further opportunities for the development of molecular detection tools and molecular fingerprinting methods will be provided. Using pyrosequencing, the detection limit of 0.1% relative abundance, which was the case for SSCP-fingerprinting will decrease to lower abundances, so that more rare members of the seed bank in drinking water community and relative communities will be identified. This will allow investigating the exchange of bacterial species from one microbial community to the other on a more detailed level, such as the exchange from the bulk water community to the biofilm community or from the cold water community to the hot water community. Recently, some studies used pyrosequencing for investigating drinking water biofilm in China (12, 20).

Since the study of the seasonal dynamics of the drinking water community represents the first detailed study of the community composition and its activity in a drinking water supply system (DWSS) spanning more than 1.5 years, it is of pronounced interest, if the observed variation is also found in other DWSS with different water abstraction methods. As the reservoirs showed to have a strong impact on the drinking water community, it would be interesting, to compare these results with the seasonal variation in drinking water abstracted by bank filtration or ground water, to determine the influence of these water sources on the seasonality of drinking water. Another interesting aspect of the seasonal variation in drinking water would be to focus on the investigation of genera with drinking water pathogens using genus specific primer sets producing polyvalent fingerprints. By focussing on a specific taxonomic group of bacteria, this technique allows to investigate low abundant members of the community which might have been missed when using universal community primers. Using the genus specific primer for Legionella spp. on the seasonal drinking water samples (17), we could allready show for this genus, that different *Legionella* species were present during different seasons. Further taxonomic groups of interest would be the class *Epsilonproteobacteria*, including pathogenic and potential pathogenic genera such as

Helicobacter, *Campylobacter* and *Arcobacter*. The combination of this data with epidemiological data would provide an interesting approach for the investigation of waterborne diseases.

Focussing on specific pathogens would also be interesting for samples of the biofilm communities. By screening for specific pathogens or for whole pathogenic genera, it could be investigated, which kind of biofilm enables or promotes the survival or reproduction of pathogens. As for example members of the *Clamydiales* were mainly found in a biofilm grown on PVC it could be possible to find conditions promoting such a survival. In the biofilm study, it was hypothesized that physical vicinity is more important to develop similar biofilm communities than similar surface materials. It would be of useful, to perform a long-term study on drinking water biofilms in a model drinking water distribution system, similar to the long-term study of Martiny et al. (25). In this study, different surface materials such as steel, glass, copper and PVC could be placed in different distances to each other. This setup would allow analysing the mutual influence of the respective biofilm communities over time.

Using the combined approach of SYTO9/PI staining and a subsequent FACS sorting, we were able to analyse the live and dead fraction of the drinking water community separately. It would be useful to compare these results also to other methods assessing viability of microorganisms. An interesting candidate for such a method would be the "viability PCR" (33). Using of ethidium monoacide (EMA) the nucleic acids of membrane injured cells are inactivated before NA-extraction so that only the nucleic acids of viable cells function as a template for the PCR reaction. The advantage of this method, compared to our combined approach, would be that no concentration step is needed, because the PCR can be performed directly on the environmental sample. The implementation of this method should be carefully evaluated by a combination of techniques because some bacteria are able to actively export EMA (31).

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7 Appendix

7.1 Curriculum Vitae

Karsten Henne

Biochemist

Date and Place of Birth

April 14th, 1978 — Dortmund, Germany

Academic studies

1998 - 2006	Study of Biochemistry at the Ruhr-Universität Bochum Degree: Diplom Biochemiker.
2006	Diploma Thesis at the Institute for Medical Microbiology, Prof. Dr. Sören Gatermann. Title of the Thesis: "Versuch der Identifizierung von Eigenschaften, die mit der epidemischen Ausbreitung von MRSA assoziiert sind"
2007 - 2011	PhD-Student at the Helmholtz Centre for Infection Research in the Molecular Diagnostics Laboratory, PD Dr. Manfred Höfle. Title of the thesis: "Molecular analyses of bacterial communities in drinking water and biofilms – seasonal dynamics, viability and community composition of cold and hot water"

School Education

1984 – 1988	Franziskus-Grundschule, Dortmund (primary school)
1988 – 1997	Käthe Kollwitz-Gymnasium, Dortmund, general qualification for university entrance (Abitur)

Alternative Civilian Service

1997 - 1998	Integrationsprojekt of the Vereinigten Kirchenkreise, Dortmund

Polyvalent fingerprint based molecular surveillance methods for drinking water supply systems

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ABSTRACT

Despite the relevance for public health, surveillance of drinking water supply systems (DWSS) in Europe is mainly achieved by cultivation based detection of indicator bacteria. The study presented here demonstrates the use of molecular analysis based on fingerprints of DNA extracted from drinking water bacteria as a valuable monitoring tool of DWSS and was exemplified for a DWWS in Northern Germany. The analysis of the bacterial community of drinking water was performed by a set of 16S rRNA gene based fingerprints, sequence analysis of relevant bands and phylogenetic assignment of the 16S rRNA sequences. We assessed the microflora of drinking water originating from two reservoirs in the Harz Mountains. The taxonomic composition of the bacterial communities from both reservoirs was very different at the species level reflecting the different limnological conditions. Detailed analysis of the seasonal community dynamics of the tap water revealed a significant influence of both source waters on the composition of the microflora and demonstrated the relevance of the raw water microflora for the drinking water reaching the consumer. According to our experience, molecular analysis based on fingerprints of different degrees of resolution can be considered as a valuable monitoring tool of DWSS.

Key words | drinking water bacteria, seasonal cycles, SSCP fingerprints, waterborne bacterial pathogens

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INTRODUCTION

Despite the relevance for public health, surveillance of drinking water supply systems in Europe is mainly achieved by cultivation based detection of indicator bacteria. This approach bears the risk of neglecting viable but non-culturable (VNBC) bacteria on the one hand, on the other hand, many pathogenic bacteria, including emerging ones are not monitored (Huq *et al.* 2000; OECD 2003; Watson *et al.* 2004). Careful estimates indicate that each year about 350 million people are infected by waterborne pathogens with 10–20 millions succumbing to severe cases (WHO 1997). This phenomenon is far from being restricted to developing countries but also threatens developed countries. In the USA almost 430,000 cases were reported in 126 outbreaks of waterborne infectious diseases from 1991 to 1999 (Craun *et al.* 2002).

Production of drinking water complying with international quality standards does not necessarily ensure good drinking water for the consumer (Dewettnick *et al.* 2001). Re-growth of bacteria in the distribution system is a major problem that may have adverse effects on drinking water quality and is correlated with biofilm formation. The effects of re-growth may range from effects on taste and odour to true health threats when it comes to re-growth of pathogenic bacteria (Vital *et al.* 2007). Key factors influencing re-growth of bacteria in a drinking water supply system (DWSS) are: i) concentration of organic compounds,

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ii) chlorine concentration, iii) residence time of the water in the distribution system, iv) water temperature and v) physico-chemical characteristics of the material lining the distribution pipes (Niquette *et al.* 2001).

The bacterial community of drinking water plays a crucial role for the drinking water quality. It is the main consumer of the organic carbon in the drinking water, mineralizes it to CO_2 or other degradation products, nitrifies ammonium to nitrite and nitrate, and forms biofilms. The autochthonous microflora can sustain the growth of protozoa and metazoa (e.g. crustacean) that are visible to the consumer (Gauthier *et al.* 1999; Servais *et al.* 1995) or may have adverse effects on the taste and safety of the drinking water (Mallevialle & Suffet 1987). The microbial community of the drinking water may directly interfere with pathogenic bacteria, i.e. it can suppress or promote the survival and growth of hygienically relevant and potentially pathogenic bacteria (LeChevallier 1990). For example, the formation of biofilms enables survival or even growth of pathogenic bacteria, while the competition for the same carbon sources or the production of antibiotic substances may suppress pathogenic bacteria. Since the microbial community is a key factor of drinking water quality with respect to many aspects, its analysis is a focus of our study.

The Healthy-Water project, a project in the 6th Framework of the EU (http://www.hzi-helmholtz.de/en/healthy_water/) is aiming towards the development of new molecular detection technologies of microbial pathogens in drinking water with special emphasis on emerging pathogens (Nwachcuku & Gerba 2004). Among several approaches that are under development, fingerprint based methods and their results will be presented here, those especially have the potential to monitor the whole bacterial community and thus bear the potential to detect also unexpected pathogenic bacteria.

METHODS

Study site

The overall study comprises samples from a DWSS in Northern Germany that provides about 80 Mio m^3 of drinking water per year and is providing drinking water for about two million people. Source water of the DWSS are provided by two surface water reservoirs, an oligotrophic reservoir (Grane, pH 7.2) and a dystrophic reservoir (Ecker, pH 5.2). The collection of aerobic raw water is done from the deep water (50–58 m). More details on the DWWS are given by Eichler *et al.* 2006. The focus of this study is on tap water and the seasonal changes studied from autumn 2006 to spring 2008.

Molecular methodology

The bacterial community of the water were harvested by filtering several litres of water onto a sandwich of a glass fiber GF/F plus 0.2 μm nuclepore filter (Whatman) (for details on the molecular methods see Eichler *et al.* 2006). In brief: DNA was extracted and purified; bacterial 16S rRNA gene amplicons generated by PCR were subjected to separation by non-denaturing acrylamide gel electrophoresis enabling Single Strand Conformational Polymorphism (SSCP) analysis. DNA based SSCP analyses were performed to follow the seasonal dynamics (Schwieger & Tebbe 1998; Hoefel *et al.* 2005; Hammes *et al.* 2006). The banding patterns on the SSCP gels, used as a direct measure of the community structure, were compared by cluster analysis (GelCompare II, Applied Maths). The composition of the bacterial community was determined by sequencing the single bands of the gel pattern and identifying the sequences by phylogenetic analysis using the international 16S rRNA gene sequence data base.

RESULTS AND DISCUSSION

Overall community structure of the drinking water microflora

The overall community structure of the drinking water microflora of tap water was assessed during one and a half years at monthly intervals to understand seasonal dynamics (Figure 1). These DNA based community fingerprints are banding patterns of single 16S rRNA genes separated according to sequence differences using SSCP electrophoresis. Ideally, the single bands represent different bacterial

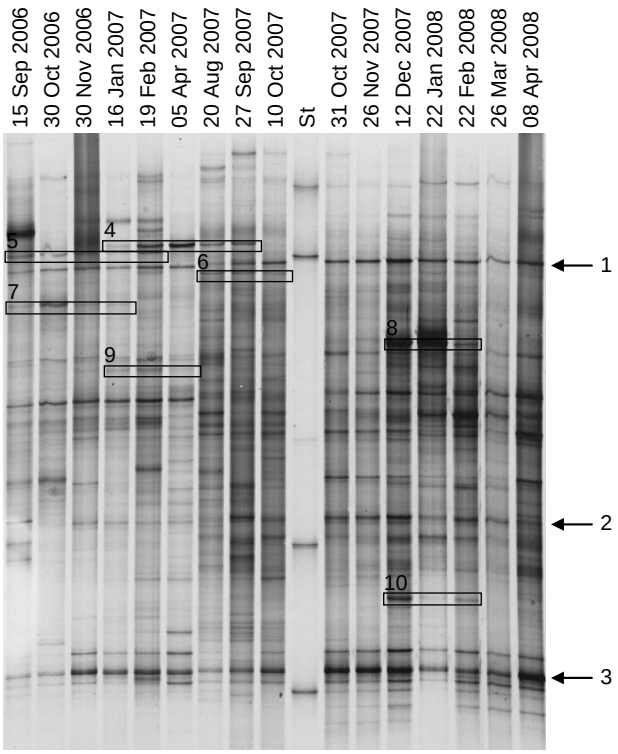

Figure 1 | DNA based community fingerprints from tap water samples obtained at the indicated dates. Arrows indicate bands observed in all samples, bands in boxes are only observed during certain times of the year. St = standards of reference bacterial species.

taxa at about the species level (Schmalenberger *et al.* 2001). For a detailed analysis of the single banding patterns density curves were produced using an electronic scanner (Figure 2). These density patterns show peaks, corresponding to the specific bands, and allow quantification of the amount of single strand DNA present in the single bands by integrating the area under the specific peak. A first comparison of the fingerprints shows that there are three

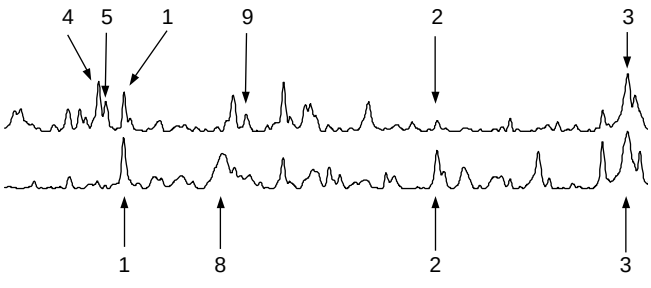

Figure 2 | Density curves from the banding pattern of the community fingerprints from two different samples (constant bands 1–3 in bold). Band numbering is consistent with Fig. 1.

major bands (1–3 marked with arrows in Figure 1) that occurred in all samples whereas several bands occurred only during certain times of the year (boxes 4–10 in Figure 1). The banding patterns of the single drinking water communities comprise about 40 to 80 different bands above the relative abundance threshold of 0.1% of the total DNA per lane. The constant bands represent 6–24% of the total DNA per lane leaving about 59–87% of the DNA for the variable bands. A seasonal pattern of the three constant bands can be recognized by comparing their relative amounts (Figure 3). Especially the most abundant band 3 shows a strong increase, from 3.6 to 16%, in October and a decline in January to March in both winters studied. Overall, these constant bands can be assumed to represent three different bacterial species that showed seasonal changes in their relative abundances by a factor of four according to the DNA abundance of the band. For a detailed understanding of the variation in the banding patterns, i. e. the community structure of the whole bacterial drinking water microflora, a cluster analysis was performed that allows a statistical comparison of the banding patterns of the different lanes (Figure 4). The cluster analysis revealed that the banding patterns changed in about 3 to 4 month intervals as revealed by the six main clusters in Figure 4. In addition, the cluster late summer 07 forms a subgroup with cluster autumn 07 as well as cluster winter 08 with spring 08. This sub-grouping indicates that the bacterial microflora is continuously changing, but mostly still related to the previous microflora.

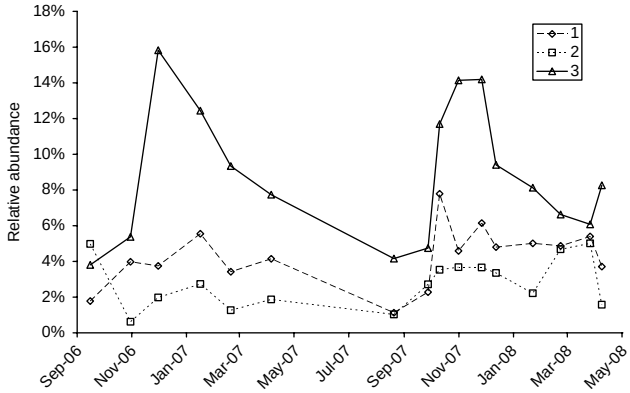

Figure 3 | Seasonal variation of the relative abundances of the single strand DNA of the three major bands (1–3) representing three different bacterial species.

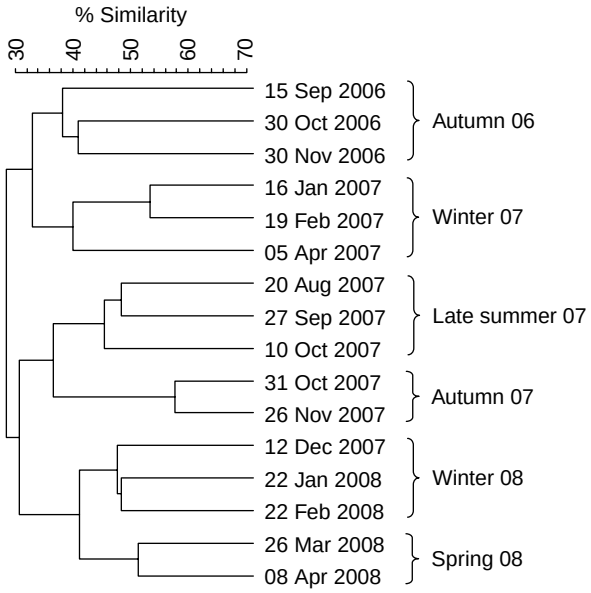

Figure 4 | Cluster analysis of all banding patterns from the community fingerprints shown in Fig. 1 (analysis was done by using GelCompare II (Applied Maths), Algorithms: Dice, Complete Linkage, all bands above 0.1% abundance included in analysis).

Taxonomic composition of the drinking water microflora

For identification of the single bacterial taxa represented by the bands of the community fingerprints, these bands have to be excised and sequenced. The generated 16S rRNA partial sequences (about 420nt) can then be compared with the large data set of bacterial 16S rRNA sequences available in international databases to identify the closest known bacterial species. In a previous study of the same DWSS, we identified 71 unique phylotypes, i. e. 16S rRNA gene sequences with a sequence similarity of $>$ 98% and phylogenetic uniqueness as discrimination criteria that comprised most of the bacterial species in this drinking water community (Eichler *et al.* 2006). Using these phylotypes as a reference data base, we could identify the three constant bands as the following bacterial taxa: band 1 = *Methylophilus* sp. (identical to phylotype 1 from Eichler *et al.* 2006, class *Betaproteobacteria*); band 2 = identical to phylotype 21 from Eichler *et al.* 2006, phylum *Actinobacteria*); band 3 = identical to phylotype 22 from Eichler *et al.* 2006, class *Alphaproteobacteria*). All three phylotypes belonged to bacterial species that have not been cultured and could only be identified by molecular analysis of DNA extracted from drinking water. In addition, all three phylotypes belonged to different bacterial classes or phyla indicating a large phylogenetic diversity of the drinking water microflora (Williams *et al.* 2004). As pointed out above, several bands (number 4 to 10) occurred only during a specific period and can be seen as indicators of changes in the structure and composition of the drinking water microflora. Sequence comparison of band 8 revealed that it was identical with phylotype 6 from Eichler *et al.* 2006 representing a betaproteobacterium from the genus *Simonsiella*. This phylotype had only been observed before in the dystrophic Eker reservoir microflora and can therefore be considered as an indicator for this microflora.

The analysis of the bacterial community by SSCP fingerprints has already been shown to be of great use for the study of the impact of the source water and the water treatment processes on the drinking water bacterial community. Eichler *et al.* (2006) have shown that the bacterial community structure of the raw water samples from the two reservoirs was very different reflecting the different limnological conditions of the reservoirs (highly dystrophic vs oligotrophic reservoir). No major changes of the structure of the bacterial community were observed after flocculation and sand filtration, while chlorination of the processed raw water strongly affected bacterial community structure as best reflected by the RNA-based fingerprints. According to assessment of the community composition by sequencing of abundant bands and phylogenetic analysis of the sequences obtained, the taxonomic composition of the bacterial communities from both reservoirs was very different. After chlorination, growth of nitrifying bacteria was observed. Detailed analysis of the community dynamics of the whole DWSS revealed a significant influence of both source waters on the composition of the microflora and demonstrated the relevance of the raw water microflora for the drinking water provided to the end user.

CONCLUSIONS

- The DNA based community fingerprints allowed to follow the seasonal dynamics of the whole bacterial microflora in tap water.

- The SSCP fingerprints enabled the assessment of the relative abundance of all bacterial members of the drinking water microflora to a threshold of 0.1% relative abundance and, after sequencing, their taxonomic identification to the species level.
- The seasonal dynamics of the tap water microflora was characterized by three constant and 40–80 varying members of the bacterial community.

These insights into the bacterial community dynamics of a drinking water supply system obtained during this and the former study led us to recommend molecular analysis based on fingerprints of different degrees of resolution as a valuable monitoring tool of DWSS. The rapid overview gained on the DWSS bacterial community can be furthermore improved and accelerated by standardized formats of the molecular analysis.

Future perspectives and applications of fingerprints as tools for drinking water research and monitoring

In the future, SSCP analysis can be used to focus on specific pathogenic bacterial groups of interest what is currently under development in the Healthy-Water project. To achieve this goal, primers with a different degree of specificity are designed and applied to generate fingerprints for pathogenic bacterial genera or species of interest such as *Campylobacter, Arcobacter* and *Helicobacter* (Moreno *et al.* 2004; Sandberg *et al.* 2006). Especially, with respect to biofilms, analysis of DWSS for these genera are of high relevance to human health (Juhna *et al.* 2007; Watson *et al.* 2004).

In many cases a higher phylogenetic resolution is needed than the one retrievable from the fingerprint band sequences in order to get a more precise taxonomic position of the target pathogenic bacterium. An improvement of the phylogenetic resolution can be achieved by designing highly specific primers and probes of a different degree of specificity based on the sequence of bands of interest (Höfle *et al.* 2005). Using these highly specific primers allows the generation of a complete 16S rRNA gene sequence (>1400 nucleotides) of aquatic bacteria (Höfle *et al.* 2005). This full 16S rRNA sequence allows a more precise analysis of the phylogenetic affiliation compared to the fragments obtained from the SSCP gel (about 420 nucleotides). Additionally, quantification of specific (pathogenic) bacteria by real-time PCR can be linked to SSCP-fingerprints. The above mentioned primers designed based on the fingerprint band sequences can be used for real-time PCR. This is of specific relevance when a new organism is detected by fingerprints that are of interest, e.g. suspicious to be a pathogenic or noxious bacterium, but not yet cultivated and the 16S rRNA sequence is not yet available in public data bases. These examples demonstrate the great potential of molecular fingerprint analyses for an improved monitoring of DWSS and a better understanding of possible hygienic risks related to various treatment and management procedures.

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MICROBIOLOGY OF AQUATIC SYSTEMS

Assessing the Viability of Bacterial Species in Drinking Water by Combined Cellular and Molecular Analyses

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Abstract The question which bacterial species are present in water and if they are viable is essential for drinking water safety but also of general relevance in aquatic ecology. To approach this question we combined propidium iodide/SYTO9 staining ("live/dead staining" indicating membrane integrity), fluorescence-activated cell sorting (FACS) and community fingerprinting for the analysis of a set of tap water samples. Live/dead staining revealed that about half of the bacteria in the tap water had intact membranes. Molecular analysis using 16S rRNA and 16S rRNA gene-based single-strand conformation polymorphism (SSCP) fingerprints and sequencing of drinking water bacteria before and after FACS sorting revealed: (1) the DNA- and RNA-based overall community structure differed substantially, (2) the community retrieved from RNA and DNA reflected different bacterial species, classified as 53 phylotypes (with only two common phylotypes), (3) the percentage of phylotpes with intact membranes or damaged cells were comparable for RNA- and DNA-based analyses, and (4) the retrieved species were primarily of aquatic origin. The pronounced difference between phylotypes obtained from DNA extracts (dominated by *Betaproteobacteria*, *Bacteroidetes*, and *Actinobacteria*) and from RNA extracts (dominated by *Alpha-*, *Beta-*, *Gammaproteobacteria*, *Bacteroidetes*, and *Cyanobacteria*) demonstrate the relevance of concomitant RNA and DNA analyses for drinking water studies. Unexpected was that a comparable fraction (about 21%) of phylotypes with membrane-injured cells was observed for DNA- and RNA-based analyses, contradicting the current understanding that RNA-based analyses represent the actively growing fraction of the bacterial community. Overall, we think that this combined approach provides an interesting tool for a concomitant phylogenetic and viability analysis of bacterial species of drinking water.

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Introduction

Drinking water commonly provides a diverse microflora to the end user despite the fact that water processing eliminates a large fraction of microorganisms present in raw water, as shown by detailed molecular studies [14, 34]. Bacteria originating from source water, regrowth in bulk water and biofilms of the distribution network which contribute to the generation of a diverse bacterial community in drinking water [17].

Molecular methods, such as 16S rRNA-based and 16S rRNA gene-based fingerprints, can provide an overview on the bacterial community and thus can overcome the restriction of cultivation-based methods that detect only the few bacteria growing under the respective cultivation conditions [9]. These molecular methods allow overcoming the problem of non-culturability for viable-but-non-culturable bacteria, i.e., even under adequate cultivation conditions, these bacteria do not grow due to physiological constraints [21]. However, molecular methods based on extracted

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nucleic acids cannot distinguish between live and dead bacteria [6, 31]. During the last years, a broad set of fluorescent stains was developed allowing insight into the physiological state of bacteria [22]. Stains assessing membrane integrity, such as propidium iodide (PI) and SYTO9, are considered to distinguish between membrane-intact and membrane-injured cells [7]. This staining procedure has been evaluated and compared by a set of studies to other staining procedures for assessment of the physiological state of the bacteria [4, 11, 22]. Membrane injury was evaluated as a reliable criterion for cell death where recovery is highly unlikely.

Bacterial community fingerprints and subsequent sequencing of the single fingerprint bands followed by phylogenetic analysis can provide an overview on the structure and composition of bacterial drinking water communities [14]. Besides providing an overview, fingerprints allow the study of any bacterial taxon in a community if specific primers are used to better understand its ecology [19] that is of special relevance for pathogenic taxa. 16S rRNA-based fingerprint analyses can be based on the analysis of environmental DNA or RNA. In general, it is assumed that RNA-based fingerprints represent the active part, especially the actively growing part, of the bacterial community whereas DNA-based analyses provide insight into the bacterial members present in the community [14, 29]. Since viability is a major issue for drinking water bacteria, the comparison of DNA- and RNA-based analyses is of great interest. Combining these DNA- and RNA-based fingerprint analyses with the distinction for membrane integrity was intended to provide new insights in the bacterial microflora and its viability.

Today's drinking water quality assessment is still based on the culture-based detection of indicator bacteria, i.e., *Escherichia coli* or fecal enterococci. Though molecular methods could provide better insights into the bacterial community, it is crucial to include the aspect of viability in the molecular methods used. To this end, we developed a procedure that combined the advantages of culture-independent molecular methods and the discrimination of membrane-intact and membrane-injured cells provided by the viability stains. Using FACS, the membrane-intact ("live") and membrane-injured cells ("dead") were separated and afterwards analyzed by community fingerprinting. The aim of our study was to elucidate by this approach which bacterial taxa are alive in finished drinking water. Both nucleic acids, DNA and RNA, were extracted from three fractions, i.e., total, "live", and "dead", and analyzed by 16S rRNA-based and 16S rRNA gene-based SSCP fingerprinting followed by sequencing of the fingerprint bands to provide insight into the taxonomic composition of the bacterial community. The study was encouraged by a previous analysis of the RNA-based bacterial community structure of drinking water that showed the proof of principle of the technical approach [24]. This previous study indicated the need of a direct comparison of DNA and RNA community structure and a detailed phylogenetic analysis that are now provided. In the present study, differences between DNA- and RNA-based fingerprints were analyzed to gain information about the active vs. present part of the bacterial drinking water microflora in the light of membrane integrity. To our knowledge, this is the first study that applies both, DNA- and RNA-based community analysis up to the species level combined with FACS sorting based on live/dead staining. This allowed a comparison of present, "live" and "dead" bacterial species for RNA and DNA extracts.

Material and Methods

Study Site and Sampling

Drinking water samples were obtained on 3 days, i.e., 25 March 2008 (sampling A), 31 March 2008 (sampling B), and 5 May 2008 (sampling C) from the tap in lab D0.04 of the Helmholtz Centre for Infection Research (HZI), Braunschweig-Stöckheim, Germany. Sampling A and B were taken as samples where a high similarity was expected due to the short time interval, sampling C was considered to display a distinct community due to the previously observed seasonal changes [19]. The drinking water originated from two surface water reservoirs (oligotrophic and dystrophic water) situated in a mountain range 40 km south of Braunschweig. Water processing included flocculation/coagulation, sand filtration, and chlorination (0.2–0.7 mg l^{-1}). In 2008 and 2009 no chlorine was detected at the sampling point at the HZI by using the colorimetric test "Aquaquant Chlor" from Merck for detection of free and total chlorine (detection limit 0.01 mg/l). More details on the respective drinking water supply system are given elsewhere [14].

For live/dead staining and FACS, drinking water microorganisms were concentrated 100–400-fold. Eighteen liters of drinking water were filtered onto a 0.2-μm pore size polycarbonate filter (90 mm diameter; Nucleopore; Whatman, Maidstone, United Kingdom), scraped, and washed off from the filter carefully with 25 ml of 0.9% NaCl in sterile water (Fig. 1). A part of the biomass was either immediately used for the staining procedure as indicated below, and an aliquot was immediately frozen for later molecular analysis (−70°C).

For comparing the impact of concentration on the drinking water microflora, the drinking water microorgan-

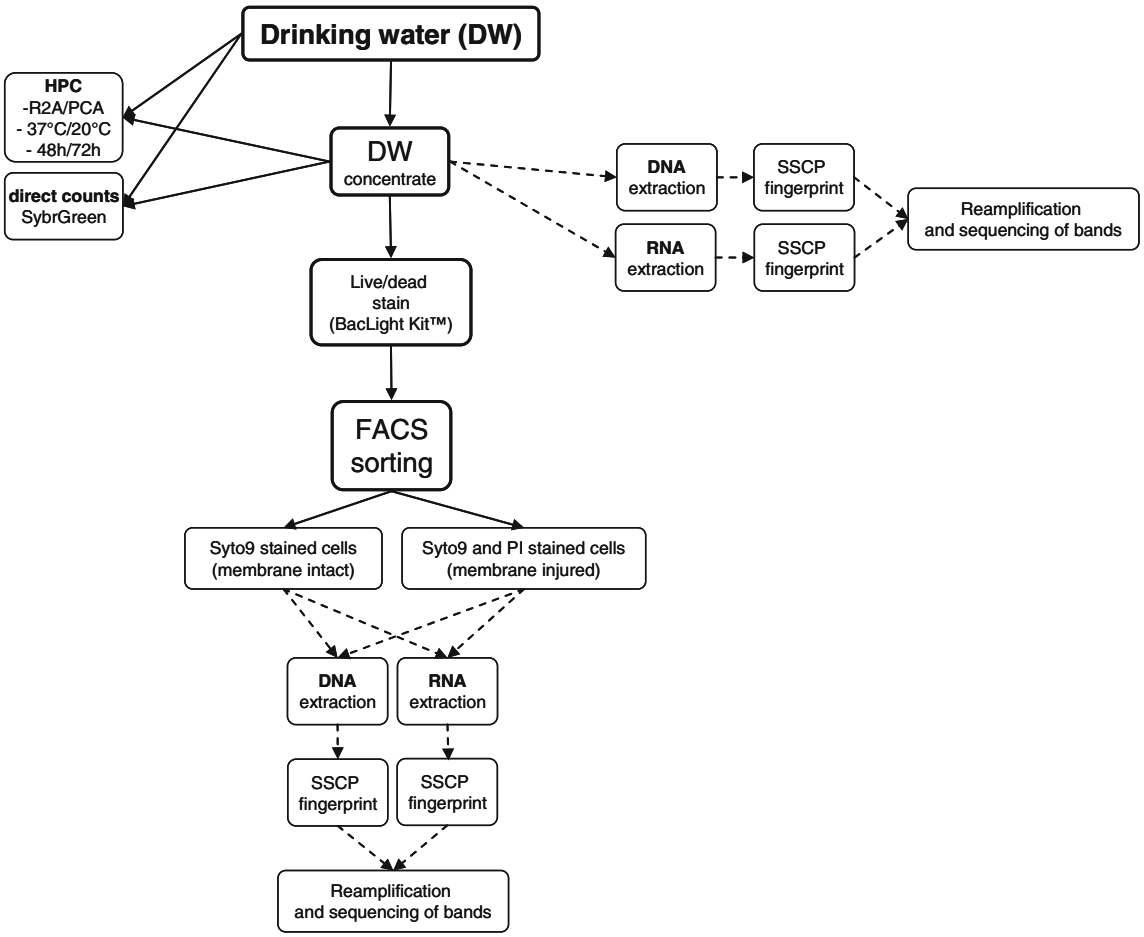


Figure 1 Flow chart of the combined analysis of drinking water samples using FACS and SSCP fingerprinting. Eighteen liters of drinking water were filtered onto a 0.2-μm Nucleopore filter, scraped, and washed off the filter with 0.9% saline solution. The drinking water bacteria were stained with the BACLight Kit™ for 20 min in the dark. After cell sorting, the differently stained fractions were analyzed by molecular methods (*dashed lines*), i.e., nucleic acids (DNA and RNA) were extracted and subjected to SSCP analysis. Sequence information was gained by reamplification and sequencing of single bands. This overall procedure is termed as "FACS sorting experiment", and was performed for every sample analysis ("sorting A-C") using the water of sampling dates A-C, respectively

isms were additionally harvested by our routine procedure, i.e., filtration of 5 l of drinking water on a filter sandwich consisting of a 0.2-μm pore size polycarbonate filter (90 mm diameter; Nucleopore; Whatman) with a precombusted glass fiber filter on top (90 mm diameter; GF/F; Whatman) [13]. Filter sandwiches were stored at −70°C until further analysis. Per sampling date, five sandwich filters were obtained.

Staining and Enumeration of Drinking Water Bacteria

Total bacteria from formaldehyde-fixed samples (2% final concentration) were stained with Sybr Green I dye (1:10,000 final dilution; Molecular Probes, Invitrogen) for 15 min at room temperature in the dark. Five-milliliter portions were filtered onto 0.2-μm pore size Anodisc filters (Whatman) and mounted with Citifluor on microscopic glass slides [35]. Slides were either analyzed directly with epifluorescence microscopy or stored frozen (−20°C) until examination. For epifluorescence microscopy, a microscope (Axioplan, Zeiss) with suitable fluorescence filters was used and the slides were examined using 100-fold magnification. For each filter, either ten photographs were taken and image sections of defined size (0.642×0.483 mm) were analyzed using the Image J software from MacBiophotonics (http://www.macbiophotonics.ca/) or 30 fields (0.125×0.125 mm) were counted by eye.

Heterotrophic Plate Counts

Heterotrophic plate counts (HPC)s were done in triplicate using an aliquot of the drinking water concentrate and the spread plate technique on either R2A agar (Oxoid) or tryptone soy agar (Oxoid) plates. Incubation was carried out at two different temperatures according to the German drinking water ordinance (36°C for 48 h and 22°C for 72 h) (Verordnung über die Qualität von Wasser für den menschlichen Gebrauch (Trinkwasserverordnung—TrinkwV

2001) Geändert durch Art. 363 V v. 31.10.2006 I 2407, 2001).

Concentrating, Live/Dead Staining and FACS Analysis of Drinking Water Bacteria

For FACS, the concentrated biomass of the drinking water samples was stained for subsequent FACS analysis with SYTO 9 and propidium iodide (PI) (final concentrations 5 and 30 μM, respectively; BacLight Kit, Molecular Probes [18]) according to the prescription of the manufacturer. After an incubation time of 20 min in the dark, cells were subjected to FACS sorting using a MOFLO cytometer (Beckman Coulter, Krefeld, Germany) with a 488-nm laser. The band pass filters used were 530/40 and 616/26 nm for SYTO 9 and PI, respectively.

Nucleic Acid Extraction from Drinking Water and Sorted Fractions

DNA and RNA were extracted from the filter sandwiches and the concentrates of the drinking water samples; the latter were analyzed before and after staining and FACS sorting as described above. For extraction of DNA and RNA, a modified DNeasy/RNeasy protocol (Qiagen, Hilden, Germany) was used. In this procedure, sandwich filters were cut into pieces, incubated with lysis buffer containing 10 mg/ml lysozym (Sigma) for 30 min (DNA) or 20 min (RNA) in a 37°C water bath. After a mechanical homogenization by shaking with glass beads, the samples were heated to 70°C in a water bath for 20 min (DNA) or 15 min (RNA). After filtration through a polyamide mesh with 250-μm pore size, absolute ethanol was added to the filtrate (ratio filtrate/ethanol 2:1) and the mixture was applied to the adequate spin-column of the kit. After this step, the protocol was applied according to the manufacturer's instructions. For the RNA, a subsequent on-column DNase digestion was applied. Nucleic acids were eluted from the columns with DNase/RNase free water and stored at −20°C. The nucleic acids were quantified using Ribogreen (RNA or ssDNA quantification, Molecular Probes; Invitrogen) or Picogreen (dsDNA quantification, Molecular Probes; Invitrogen) according to [36].

For extraction of the nucleic acids from the concentrated or the sorted fractions of microorganisms (considered as dead or alive), 1–2-ml portions of the concentrates before and after sorting were harvested by centrifugation for 15 min at 15,000×*g*. The pellets were either frozen or directly used for nucleic acid extraction using the DNeasy/RNeasy protocol (Qiagen). Pellet supernatant was checked by epifluorescence microscopy for microorganisms; in no case cells were observed. DNase digestion for the RNA was applied as described above.

16S rRNA and 16S rRNA-Gene Based Community Fingerprints

PCR amplification of 16S rRNA and of its respective genes from the extracted nucleic acids were performed using the previously described primers COM1 (5′-CAGCAGCCGCGGTAATAC-3′) and COM2 (5′-CCGTCAATTCCTTTGAGTTT-3′), amplifying positions 519 to 926 of the *E. coli* numbering of the 16S rRNA gene [33]. For single-strand separation, a 5′-biotin-labeled forward primer was used according to [14]. From RNA, a reverse transcription was carried out before PCR using the First Strand cDNA synthesis Kit (Fermentas) following the manufacturer's recommendations. Each amplification was carried out using 2 ng DNA/cDNA template in a final volume of 50 μl, starting with an initial denaturation for 15 min at 95°C. A total of 30 cycles (30s at 95°C, 30s at 55°C, and 1 min at 72°C) was followed by a final elongation for 10 min at 72°C. Amplification was achieved using HotStarTaq DNA polymerase (Qiagen).

For the preparation of ssDNA and community fingerprints, a variant of the protocol described by Eichler et al. [14] was applied. Briefly, magnetic streptavidin-coated beads (Promega, Madison, WI, USA) were applied to obtain ssDNA from the PCR amplicons. Quantification of the obtained ssDNA was performed on a 1.5% agarose gel by comparison with a low-molecular-weight marker (Invitrogen low-DNA-mass ladder). For SSCP fingerprinting analysis, 25 ng of the obtained ssDNA was mixed with gel loading buffer (95% formamide, 10 mM NaOH, 0.25% bromphenol blue, 0.25% xylene cyanol) in a final volume of 7 μl. After incubation for 3 min at 95°C, the ssDNA samples were stored on ice, loaded onto a nondenaturing polyacrylamide-like gel (0.6× MDE gel solution; Cambrex BioScience, Rockland, ME) and electrophoretically separated at 20°C at 400 V for 18 h on a Macrophor sequencing apparatus (Pharmacia Biotech, Germany). The gel was silver stained according to the method described by [2]. Dried SSCP gels were digitized using an Epson Expression 1600 Pro scanner, bands with an intensity of >0.1% of the total lane were considered for further statistical analysis. Similarity coefficients were calculated using Pearson correlation algorithm. Dendrograms were constructed using the Unweighted Pair Group Method with Arithmetic Mean using the GelCompare II software (Applied Maths, Kortrijk, Belgium).

Reamplification and Sequencing of ssDNA Bands from SSCP Fingerprints

Sequence information was obtained following the protocol of Eichler et al. [14]. Briefly, ssDNA bands were excised from the SSCP acrylamide gels, and boiled in Tris buffer (10 mM Tris-HCl, 5 mM MgCl2, 5 mM KCl, 0.1% Triton

X-100, pH 9). Seven microliters of the solution was used in a reamplification PCR with the unbiotinylated COM primers described above. After checking the PCR amplicons on a 2% agarose gel, the amplicons were purified and subsequently sequenced by cycle sequencing (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit; Applied Biosystems, Foster City, CA, USA). Before analysis on an ABI Prism 3100 Genetic Analyzer, the products were purified using the BigDye Terminator purification kit (Qiagen). Phylogenetic identification of the sequences was done either by the NCBI Tool BLAST/blastn [1] for comparison with the closest 16S rRNA gene sequence or the Ribosomal Data Base Project Seqmatch Tool [10] for the identification of the closest described relative (Gene Bank Data base until September 9, 2009). To define a phylotype, we chose two definite sequence differences on a mean stretch of 300-bp sequence length as criterion. The partial 16S rRNA gene sequences retrieved from the fingerprints are accessible at the GenBank/EMBL/DDBJ accession numbers GQ 917122-GQ 9171174.

Results

Bacterial Cell Counts and Heterotrophic Plate Counts

The results on the bacterial counts are detailed in Fig. 2. For drinking water samples obtained from the tap at the three sampling dates, the total bacterial cell numbers were in the range of 3 to 4×10^5 cells ml^{-1}; in the concentrates (100- to 400-fold) of the drinking water bacteria used for viability staining, the cell numbers ranged from 5.1×10^7 to 1.2×10^8 cells ml^{-1}. After staining with PI and SYTO9, the fraction of membrane-intact cells determined microscopically accounted for 53±6% of the total bacteria while the membrane-injured fraction accounted for 47±6%. Heterotrophic plate counts (HPC) made from the concentrates were on average substantially less than the total bacterial counts, i.e., four to five orders of magnitude depending on medium and incubation time. HPCs on R2A agar at 22°C and after 72 h exceeded all plate counts on the other media and temperatures, and ranged from 2.0 to 4.1×10^3 CFU ml^{-1} in the concentrate. For the not concentrated tap water, between 3 and 31 CFU ml^{-1} were detected.

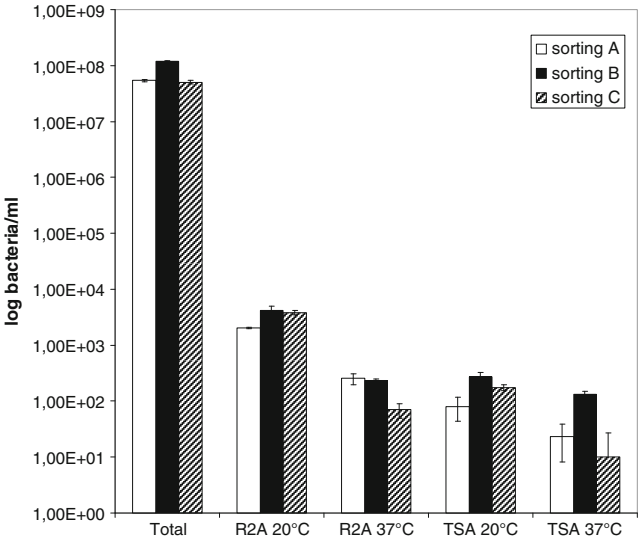


Figure 2 Total bacterial cell numbers of the drinking water concentrate used in the three FACS sorting experiments sorting A (25 March 08, *open bars*) sorting B (31 March 08, *black bars*) and sorting C (5 May 08, *hatched bars*). Total bacterial counts were determined by epifluorescence microscopy using Sybr Green I staining of formaldehyde-fixed samples. Heterotrophic plate counts were determined using 1 ml (or appropriate dilutions) concentrated drinking water and the spread plate technique on the media and temperatures indicated. *Error bars* represent standard deviation of at least three replicates

FACS Results of PI/SYTO-Stained Drinking Water Bacteria

After PI/SYTO staining, drinking water bacteria were analyzed based on two scatter parameters (forward and side scatter) and the fluorescence signal. For the analysis, some particles were excluded due to a lower forward scatter signal indicating particles or cell debris with little or no DNA content (Fig. 3a). After staining, the majority (around 70-80%) of all cells could be sorted into two fractions, i.e., non-membrane-injured SYTO9-positive cells and membrane-injured PI-positive cells (Fig. 3b). Subsequent purity control as well as a check by epifluorescence microscopy demonstrated the effectiveness of the sorting (Fig. 3c, d). Flow cytometric analysis of the drinking water bacteria, based on comparison with reference beads of defined sizes, indicated that all fractions of microorganisms (total, SYTO9 positive, PI positive) had a narrow size distribution and a rather small diameter, i.e., on the average 0.69 μm (c_v, 1.3%; data not shown). In the three sorting experiments A-C (corresponding to samplings A-C), total cell numbers recovered from FACS ranged around 10^6 cells per fraction (membrane intact, membrane injured) that were subsequently subjected to nucleic acid extraction and fingerprinting.

Structure of the Bacterial Community of Drinking Water Before and After Sorting

DNA- and RNA-based 16S rRNA SSCP fingerprints were used to analyze the bacterial community structure and composition of the drinking water before and after the cells were sorted by FACS as membrane-intact and membrane-injured cell fractions, and to assess the effect of the concentration procedure on the bacterial community (Fig. 4). A general observation was that DNA- and RNA-based fingerprints from the same samples showed always

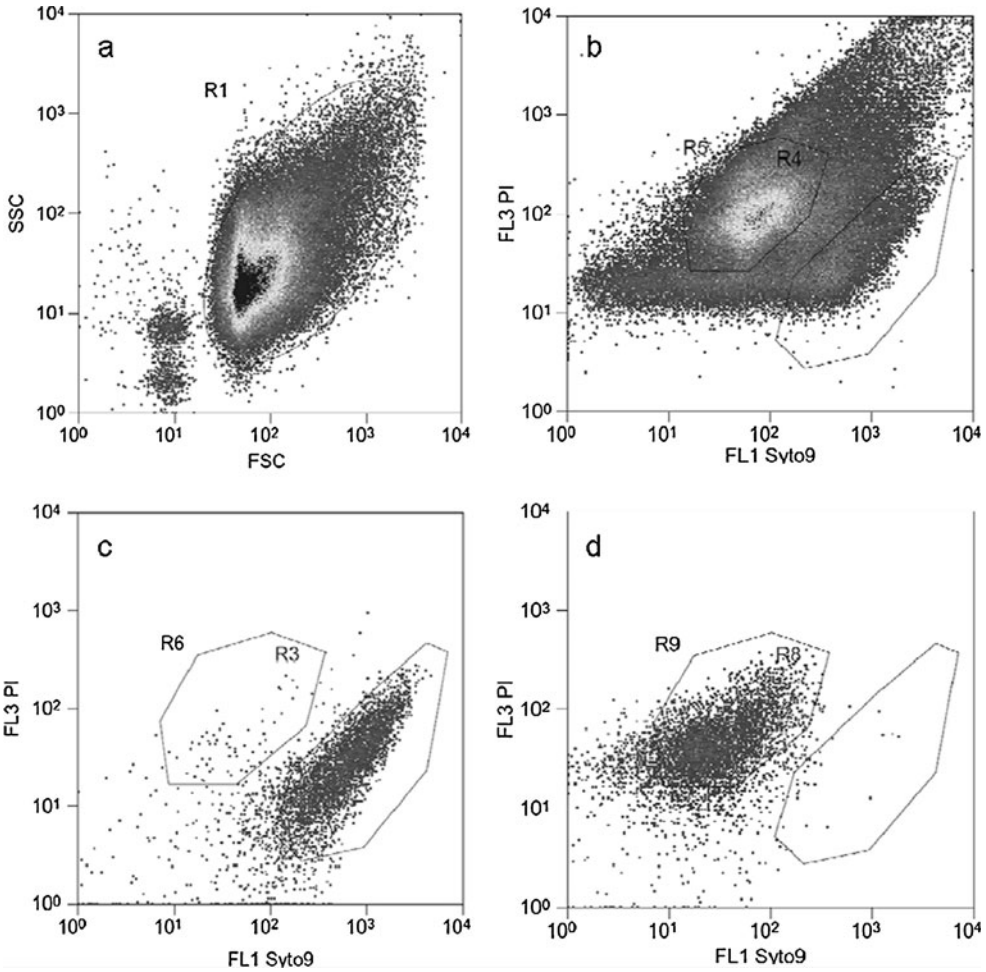


Figure 3 Results of the FACS analysis of the drinking water community. Microorganisms from 18 liters of drinking water were concentrated, stained with the BacLight Kit™ and analyzed by the flow cytometer. **a** Flow cytometric analysis of unstained cells. Cells in gate *R1* are included in the analysis and cells outside the gate were considered cell debris. **b** Flow cytometric analysis of microorganisms stained with the BacLight Kit™. Cells in gate *R4* are Syto 9 positive, cells in gate *R5* are PI positive. Purity control of the sorted fractions, **c** Syto 9-positive cells (gate *R3*) but PI negative (gate *R6*), and in **d** PI-positive cells (gate *R9*) but negative for Syto 9 (gate *R8*). Fluorescence channel, *FL1*, 530±40 nm; *FL3*, 616±16 nm; *FSC*, forward scatter; *SSC*, side scatter

very different banding patterns, a feature that was confirmed (see "Taxonomic Composition of the Different Cell Fractions") by the analysis of the species composition by sequencing of the fingerprint bands. DNA- and RNA-based SSCP fingerprints of the drinking water community with and without concentration procedure (the latter sampled on filter sandwiches) were highly comparable (see Supplementary Material Fig. 1). Fingerprints of the unsorted drinking water concentrates generated on the three sampling dates clustered closely together indicating a high similarity for the structure of the drinking water bacterial community on the three sampling dates (Figs. 4, 5). As shown in Fig. 5, the highest similarity was observed among sampling A and B for the DNA-based fingerprints (95%); the similarity among the drinking water concentrates was always higher than 76% irrespective of DNA- or RNA-based analyses or the sampling date (Fig. 5a,b).

DNA-based fingerprints of the membrane-intact and membrane-injured sorted fractions showed a very distinct pattern for each sampling day (Figs. 4a, 5a). Comparative cluster analysis of the DNA-based fingerprints showed that for each sampling date, the fingerprints from each fraction clustered more closely together than the different sampling dates, indicating that the community structure became more dissimilar among the sampling dates due to the live/dead sorting (Fig. 5a). Remarkably, after sorting the live and dead fractions of all three samplings were most closely related to each other indicating that the DNA-based fingerprints reflected often the same phylotypes as live and dead. In contrast, the RNA-based fingerprints of the sorted cell fractions showed a similar pattern among the membrane-intact fractions irrespective of the sampling date (Figs. 4b, 5b) as indicated by a tight clustering (similarity >70%, Fig. 4b). The membrane-injured sorted fractions showed a more diverse pattern for the three sampling dates, mainly caused by the large discrepancy for sampling C.

Taxonomic Composition of the Different Cell Fractions

A total of 111 bands from the DNA- and RNA-based SSCP fingerprints were sequenced to determine the taxonomic composition of the different fractions. Using a limit of ≥99% 16S rRNA gene sequence similarity as discrimination criterion, we retrieved 53 unique phylotypes for these bands (Supplementary Material Table 1). For identification,

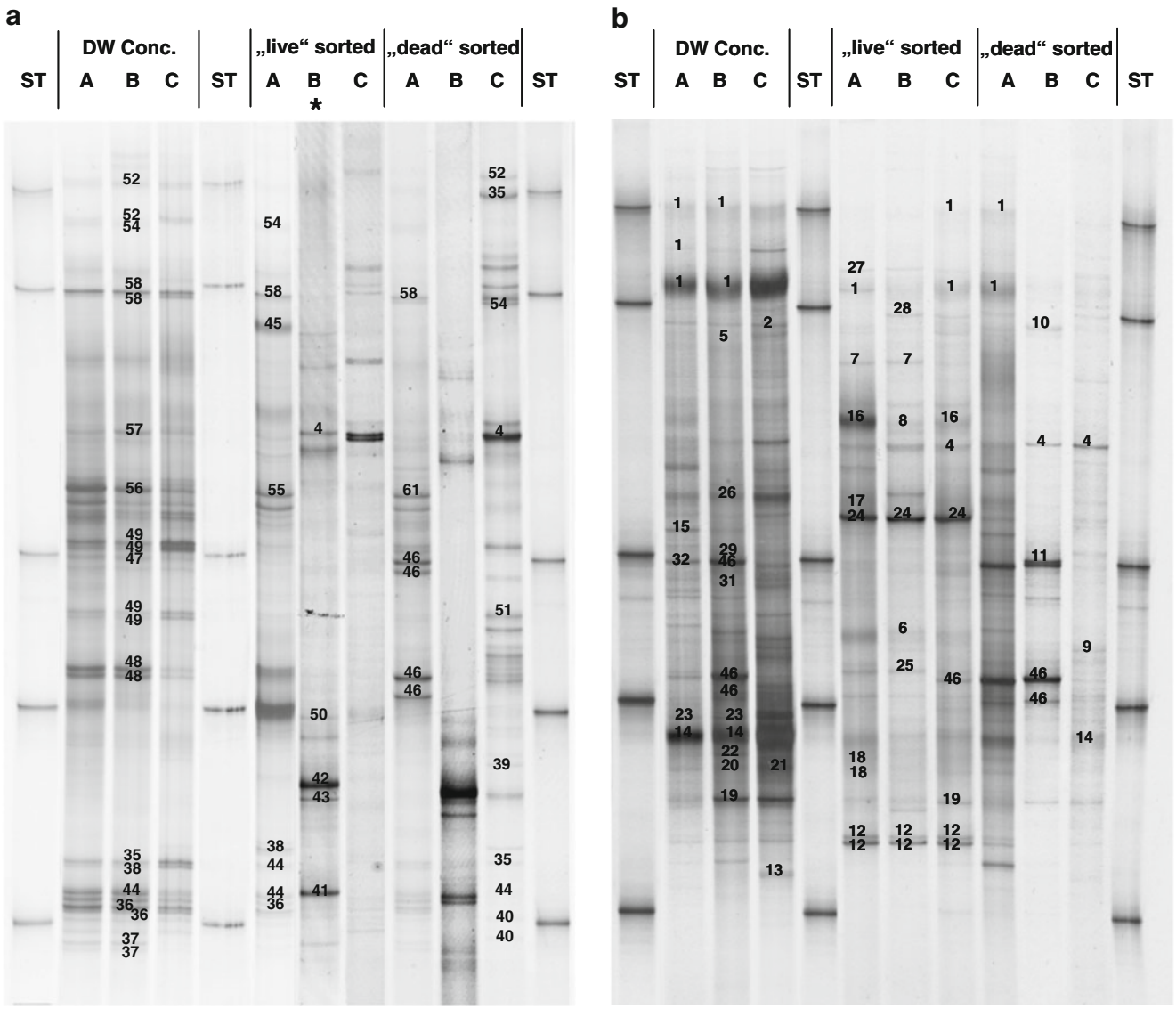


Figure 4 **a** DNA-based 16S rRNA gene SSCP fingerprints of the different FACS sorting experiments, sorting *A* (25 March 08), sorting *B* (31 March 08), and sorting *C* (5 May 08). *Numbers* represent single phylotypes of sequenced and identified bands. Phylogenetic information about these phylotypes is given in Supplementary Material Table 1. Designations of single samples, *ST*, species standard; *DW Conc*., concentrated, unsorted drinking water samples from the respective dates; membrane-intact ("*live*") sorted, SYTO9-positive fraction of the drinking water; membrane-injured ("*dead*") sorted, propidium iodide-positive fraction of the drinking water. The *asterisk* indicates a lane from a different SSCP gel. **b** RNA-based 16S rRNA SSCP fingerprints of the different FACS sampling dates. Sample designations and numbering of sequenced phylotypes are as for **a**. Phylogenetic information about the numbered phylotypes is given in Supplementary Material Table 2

the obtained sequences were compared to all databank entries in the GenBank. Out of the 53 unique phylotypes, 31 were retrieved from the RNA-based fingerprints, and 24 from the DNA-based fingerprints with only two phylotypes that were retrieved from both RNA and DNA. RNA-phylotype 1 and DNA-phylotype 52 were affiliated with the same species but were distinct by 8 nucleotides (nt) and therefore assigned to different phylotypes. Thus, the bacterial community reflected by both fingerprint types differed to a large extent.

Comparing the major taxonomic groups, the analysis of the DNA-based fingerprints (Table 1, Fig. 6a) showed that the drinking water samples were dominated by members of the *Betaproteobacteria* (eight phylotypes, with an average abundance of 14.9%), *Bacteroidetes* (seven phylotypes, 17.8%), and *Actinobacteria* (two phylotypes, 15.3%). All other classes and phyla, i.e., *Alpha-* and *Gammaproteobacteria*, *Planctomycetes*, and *Cyanobacteria*, had a low diversity (one to two phylotypes) and a low abundance (0.2–3.3%). The RNA-based

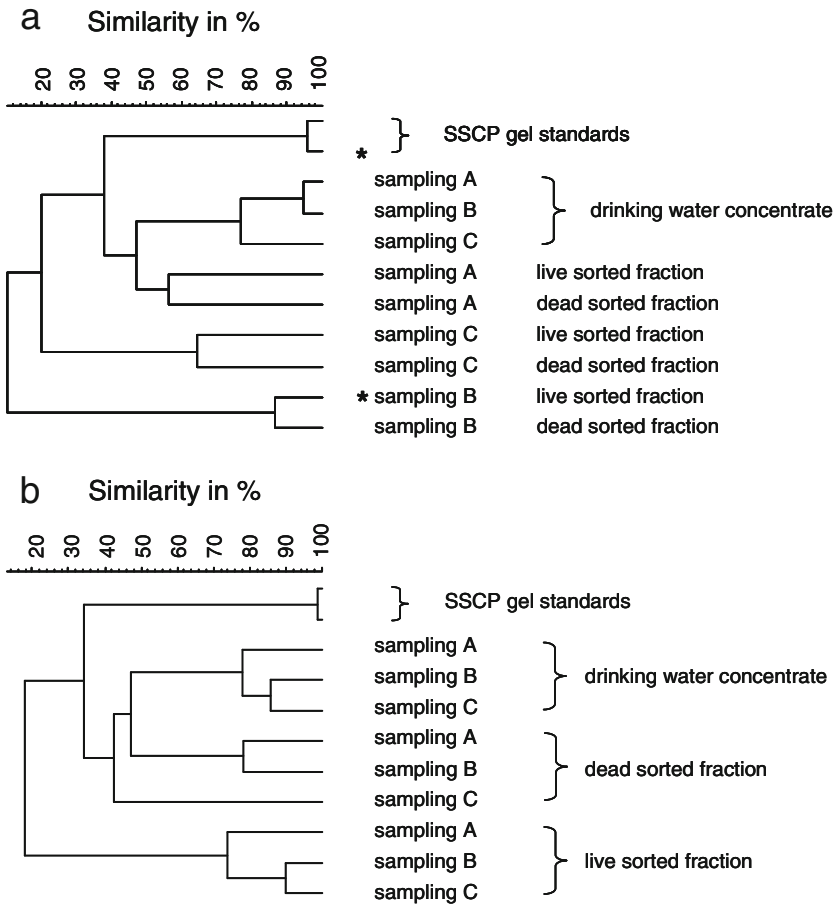


Figure 5 Cluster analysis of the two SSCP gels given in Fig. 4. Similarity coefficients were calculated using Pearson correlation algorithm. Dendrograms were constructed using the Unweighted Pair Group Method with Arithmetic mean. **a** DNA-based SSCP fingerprints of the FACS sorting experiments of the samplings *A*, (25 March 08), *B* (31 March 08), and *C* (5 May 08). Sample designations are as in Fig. 4a. The lane labeled with an asterisk is from a different SSCP gel. **b** RNA based SSCP fingerprints of the FACS sorting experiments of the different sampling dates *A*-*C*. Standards composed of five bacterial species were taken as outgroup for the cluster analysis. Sample designations are as in Fig. 4b

fingerprints (Fig. 6b) of the drinking water samples were dominated by members of the *Betaproteobacteria* (four phylotypes, 20.8%), *Cyanobacteria* (six phylotypes, 15.6%), *Alphaproteobacteria* (five phylotypes, 15.5%), *Gammaproteobacteria* (eight phylotypes, 9.5%), and *Bacteroidetes* (three phylotypes, 8.3%). The remaining four phyla, i.e., *Nitrospira, Firmicutis, Planctomycetes, Chloroflexi*, had a low diversity (one to two phylotypes) and a low abundance (0.1–4.6%). While most phyla occurred in both the RNA- and DNA-based analyses, *Actinobacteria* were never observed in the RNA-based analyses, whereas *Chloroflexi* (with a high abundance of 16% in the membrane-intact fraction of the RNA-based analyses) were never observed in the DNA-based analyses (Table 1, Supplementary Material Table 1). The single phylotypes of *Nitrospira* and *Firmicutes* also occurred only in the RNA-based analyses but had low and variable abundances (below 2.3%).

An overview on the phylogenetic diversity is shown by Supplementary Fig. 3a by a tree based on the phylogenetic analysis of the retrieved phylotypes together with the nearest cultured species. Supplementary Fig. 3a shows all occurring phyla and Fig. 3b shows the phylum *Proteobacteria* in more detail. Details of the phylogenetic analyses are listed in Supplementary Table 1. Overall, the bacterial drinking water community retrieved from RNA and DNA analyses was mostly composed of bacteria that were not related to any described species. For the DNA-based analyses, 46% of the phylotypes were not related to any described genus, 42% were affiliated with a described genus, and 38% were affiliated with a described species. For RNA-based analyses, 58% of the phylotypes were not related to any described genus, 32% were affiliated with a described genus, and 23% were affiliated with a described species. The phylotypes affiliated with a described genus were mostly members of the *Bacteroidetes*, *Alpha-*, *Beta-*, and *Gammaproteobacteria*.

From the 24 phylotypes of the DNA analyses, three phylotypes contributed to more than 5% (up to 12%) of the total (unsorted) drinking water community (Supplementary Material Tables 1 and 2). Two of these three dominating phylotypes were related to uncultured *Actinobacteria* (phylotype 48, 49). The bacterium with the highest abundance of 12.4% showed 98% similarity to the freshwater bacterium *Sediminibacterium salmoneum*, a cultured *Bacteriodetes* (phylotype 35). From the 31 phylotypes of the RNA analyses, five phylotypes contributed to more than 5% (up to 18%) of the total (unsorted)

Table 1 Abundances of the phylotypes (PT) summed up per Phyla/class displayed for the unsorted and sorted ("live/dead") fractions

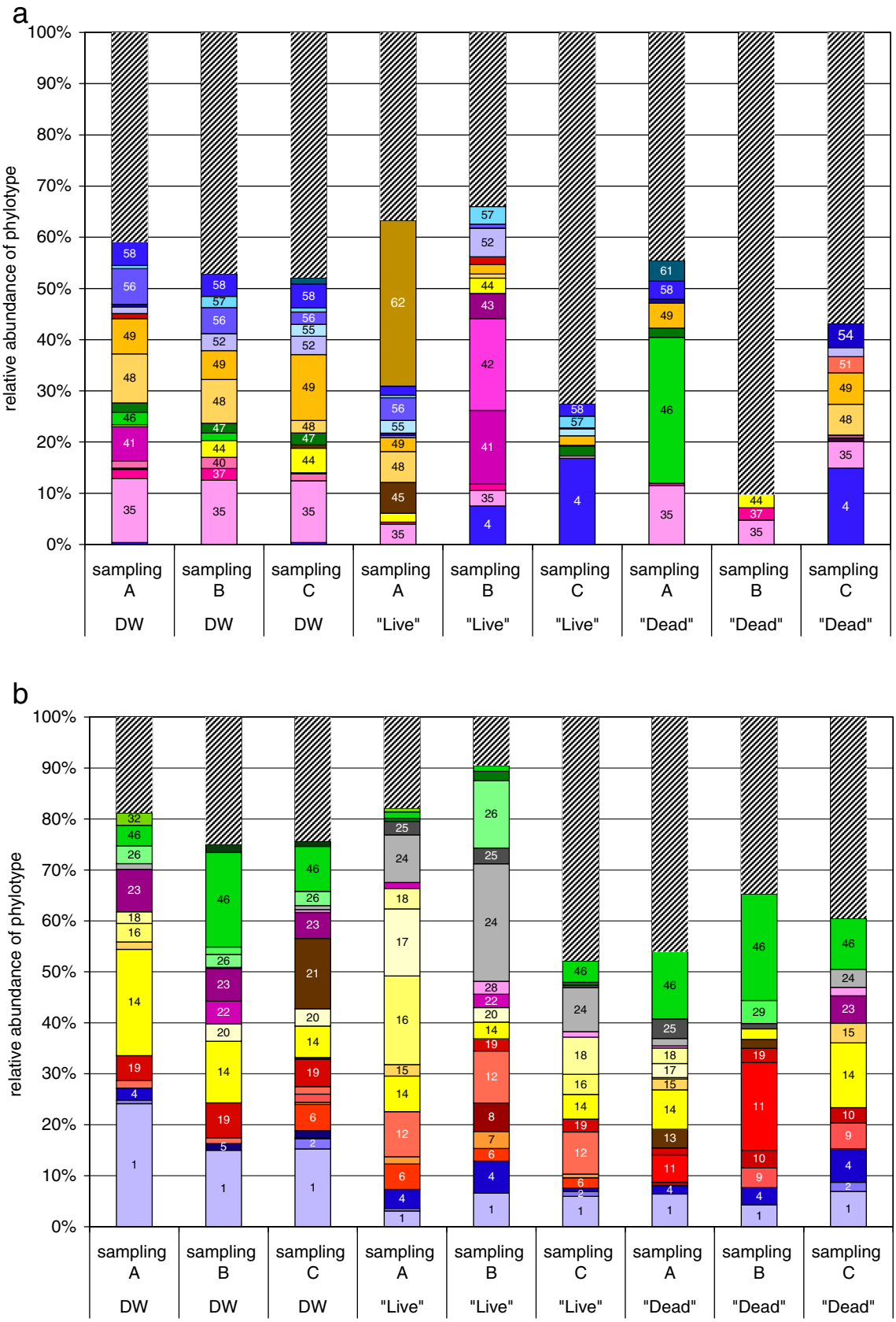
Phyla/class		All (unsorted)		Live		Dead	
	PT (*n*)	Mean A–C	SD	Mean A–C	SD	Mean A–C	SD
DNA-based analysis							
Alphaproteobacteria	1	2.83%	2.24%	1.56%	1.48%	0.92%	1.49%
Betaproteobacteria	8	14.93%	0.52%	16.82%	6.55%	9.92%	10.80%
Gammaproteobacteria	2	0.34%	0.59%	0.50%	0.86%	1.05%	1.81%
Actinobacteria	2	15.32%	1.12%	4.52%	3.71%	5.68%	6.11%
Bacteroidetes	7	17.75%	4.56%	15.45%	22.67%	8.49%	3.02%
Cyanobacteria	2	3.30%	0.97%	0.64%	1.10%	10.10%	17.49%
Planctomycetes	2	0.22%	0.38%	12.81%	22.19%	n.d.	
Identified as PTs	24	54.69%	3.83%	52.29%	21.52%	36.16%	23.66%
Percentage of PTs with only "dead" cells		20.8%					
RNA-based analysis							
Alphaproteobacteria	5	15.49%	11.41%	21.04%	20.67%	11.52%	8.19%
Betaproteobacteria	4	20.76%	5.66%	9.28%	3.14%	10.33%	4.27%
Gammaproteobacteria	8	9.50%	4.02%	17.58%	5.60%	14.27%	11.31%
Bacteroidetes	3	8.31%	2.64%	2.49%	2.31%	2.49%	3.96%
Chloroflexi	2	0.70%	0.47%	15.75%	9.10%	3.28%	2.13%
Cyanobacteria	6	15.59%	7.56%	7.85%	7.33%	16.23%	8.13%
Firmicutes	1	2.25%	1.95%	0.95%	1.64%	n.d.	n.d.
Nitrospira	1	0.10%	0.17%	n.d.		1.82%	1.84%
Planctomycetes	1	4.60%	7.96%	n.d.		n.d.	
Identified as PTs	31	77.30%	3.38%	74.93%	20.17%	59.94%	5.68%
Percentage of PTs with only "dead" cells		21.4%					

The abundances of the phylotypes are derived from the SSCP analyses of DNA and RNA extracts as shown in Fig. 4 (for details on the abundances of the single phylotypes see Supplementary Table 2). The mean of the three samplings A, B and C plus the standard deviation SD is given for the tap water sample before sorting ("All (unsorted)"), the "live" sorted fraction (cells with intact membranes), and the "dead" sorted fraction (cells with injured membranes)

PT phylotype, *n* number of phylotypes per phyla/class, *n.d.* not detected

drinking water community. These five dominating phylotypes were composed of one cyanobaterium (phylotype 46; affiliated with the genus *Synechococcus*), one gammaproteobacterium related only to uncultured bacteria (phylotype 19), one betaproteobacterium related to the species *Acidovorax facilis* (phylotype 1), one alphaproteobacterium related to the species *Bosea vestrii* (phylotype 14), and one member of the *Bacteroidetes* (phylotype 23) not related to any described genus.

All 24 DNA-based phylotypes were recovered after cell sorting in the membrane-intact and/or membrane-injured fractions indicating a recovery of 100% of the phylotypes in the sorted fractions. 38% of the DNA phylotypes occurred only in the membrane-intact fraction, 21% occurred only in the membrane-injured fraction, and 42% occurred in both fractions. Phylotypes of the major taxa *Betaproteobacteria* and *Bacteroidetes* contributed to all three fractions, i.e., membrane intact, membrane injured, and total. The two phylotypes of the *Actinobacteria* were always retrieved from the membrane-intact and membrane-injured fractions. Based on the RNA analyses, 28 of the 31 phylotypes (90%) were retrieved after sorting in the membrane-intact and/or membrane-injured fraction. From the retrieved 28 phylotypes, 32% of the RNA phylotypes occurred only in the membrane-intact fraction, 21% occurred only in the membrane-injured fraction, 46% occurred in both fractions. Phylotypes of the classes *Gammaproteobacteria*, *Cyanobacteria* and the phylum *Bacteroidetes* contributed to all three fractions, i.e., membrane intact, membrane injured, and total. All phylotypes of the *Alphaproteobacteria* were always retrieved from membrane-intact and membrane-injured fractions. Thus, the phylotypes obtained from RNA- and DNA-based analyses showed a similar ratio with respect to retrieval of their cells from the membrane-intact and -injured fractions: 32–38% had cells only in the membrane-intact fractions, 21% only in the membrane-injured fractions, and 42–46% in both fractions.


a
relative abundance of phylotype
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
sampling A DW
sampling B DW
sampling C DW
sampling A "Live"
sampling B "Live"
sampling C "Live"
sampling A "Dead"
sampling B "Dead"
sampling C "Dead"
b
relative abundance of phylotype
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
sampling A DW
sampling B DW
sampling C DW
sampling A "Live"
sampling B "Live"
sampling C "Live"
sampling A "Dead"
sampling B "Dead"
sampling C "Dead"

◀ **Figure 6** Comparison of relative abundances of the phylotypes found in the "live/dead" fractions and the drinking water concentrate (*DW*) for the FACS sorting experiments of the samplings *A-C*. **a** Phylotypes from the DNA-based SSCP fingerprints. **b** Phylotypes from the RNA-based SSCP fingerprints. *Numbers* represent the single phylotypes given in Supplementary Material Table 1a and 1b, respectively. The colors are corresponding to the major phylogenetic groups of the phylotypes, *yellow—Alphaproteobacteria*; *blue—Betaproteobacteria*; *red—Gammaproteobacteria*; green—*Cyanobacteria*; *violet—Bacteriodetes*; *brown—Planctomycetes*; *orange—Actinobacteria*; *Grey—Chloroflexi*. *Hatched bars* represent unidentified bands

After FACS sorting, major changes of the abundances of the phylotypes occurred that were far more pronounced for the DNA-based analyses than for the RNA-based analyses. Supplementary Table 1 and Supplementary Fig. 2 are providing the details on the changes of abundances with respect to the phylotypes before and after sorting, while Table 1 provides an overview on the phyla/class level. These changes of abundances through sorting were most pronounced in the membrane-intact sorted fraction for the *Chloroflexi* (phylotype (PT) 24) in the RNA-based analyses and the *Planctomyces* (PT 62) in the DNA-based analyses. Overall, we observed only few phylotypes with a high abundance in the sorted cell fractions of the DNA-based electropherograms (Supplementary Material Fig. 2a) while in the RNA-based electropherograms (Supplementary Material Fig. 2b) phylotypes with a high abundance were present in the non-sorted as well as in the sorted fractions.

For an estimate of the origin of the phylotypes, the habitat of the most similar bacterial sequence from the public data bases is given in Supplementary Table 1. Provided that the most similar sequence (1) had a similarity of higher or equal to 91% 16S rRNA gene similarity and (2) was of aquatic origin, the phylotype was rated as "of aquatic origin". Below 91% 16S rRNA gene sequence similarity the relatedness was regarded as too low to give information on the potential habitat of the phylotype. Based on these criteria, 76% of the DNA- and RNA-based phylotypes were considered as of aquatic origin which most of them from freshwater habitats. Six out of the RNA phylotypes and three out of the DNA phylotypes were not used for this assignment due to too low 16S rRNA gene sequence similarity (all these sequences had a similarity below 88% to the next sequence in the public data bases).

Discussion

Community Structure and Composition of Drinking Water Bacteria Using DNA- and RNA-Based Fingerprints

DNA- and RNA-based molecular analyses provided a very different picture of the drinking water microflora. This comprised the overall fingerprint patterns, their changes due to sorting and the retrieved phylotypes. However, the performed analyses do not precisely reflect the quantitative composition of the bacterial community. Since the amplification of 16S rRNA genes is based on PCR, a PCR bias has to be taken into account [16, 38]. According to our experience with aquatic community analysis by SSCP, the technique provides highly reproducible fingerprints of the community with high reproducibility in terms of the relative abundances of the single bands compared to the total community. Compared to real-time PCR detection of single phylotypes, low abundant phylotypes seem to be overestimated, while highly abundant phylotypes seem to be underestimated [8]. Thus, the fingerprint gives a biased but reproducible semi-quantitative picture of the bacterial community allowing comparison of different bacterial communities and observation of the dynamics of single community members.

The fingerprint analysis of the drinking water samples showed a highly consistent pattern among the three different sampling dates for both the RNA- and DNA-based analyses. A rather stable bacterial community of the investigated drinking water over time had already been shown by the seasonal study of Henne et al. [19] using DNA-based fingerprints. Though seasonal variation occurred for some members of the bacterial community, the overall community structure was rather stable during the year. The SSCP fingerprint patterns were completely different with respect to analysis of RNA and DNA of the same samples. This different pattern was confirmed by sequencing and phylogenetic analysis of the fingerprint bands. From the 24 phylotypes retrieved from the DNA-based analysis, and 31 phylotypes retrieved from the RNA-based analysis only two phylotypes (PT 4, 46) were identical, and two were affiliated with the same species (PT 1, 52). Though the same phyla with a few exceptions were detected in RNA- and DNA-based analysis, from the genus level upwards there was a pronounced divergence at the species level. This strong discrepancy between RNA and DNA-based analysis concerning the fingerprint pattern and the members of the bacterial community had already been observed by Eichler et al. [14].

Our drinking water community was dominated by phyla and classes typical for freshwater environments, i.e., *Bacteroidetes*, *Cyanobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria*. This was also the case when looking at the higher level of phylogenetic resolution, i.e., the phylotypes that were resolved approximately at the species level. The majority of the phylotypes (76%) were most closely related to sequences retrieved from aquatic habitats. This is consistent with findings of the study of the whole drinking water supply system by Eichler et al. [14]. The phylotypes identified based on the DNA-based analyses seemed to have a higher stability in the drinking water than the RNA phylotypes. 55% of the DNA

phylotypes identified in this study were also detected in the study of Eichler et al. [14] in the same drinking water supply system 5 years ago. This was different for the RNA-based phylotypes that had only a reoccurrence of 11%.

Assessment of Live and Dead Bacterial Cells Using PI/SYTO9 Staining

In our study, about half (53%) of the bacterial cells in the drinking water samples showed an intact membrane. This is in line with studies by Berney et al. [5] that reported a fraction of membrane-intact cells of about 66% in tap water that was free of chlorine as it was the case in our study. For chlorine containing tap water, Hoefel et al. [20] reported 12% membrane-intact cells for finished drinking water of an Australian water distribution system with a higher chlorination during treatment and transport, and a free chlorine residual level of 0.4 mg l^{-1} at the tap.

The propidium iodide staining is considered to provide a good estimate for membrane injury of *Bacteria* and *Archaea* [27]. In a set of studies, this staining procedure has been evaluated and compared with other staining procedures for assessment of the physiological state of the bacteria [15, 22]. Besides the evaluation of methodological aspects, recent studies were done for drinking water with added bacteria and the indigenous microflora. Berney et al. [3] tested PI for *E. coli* in drinking water submitted to UV and sunlight irradiation using a set of different viability stains. The study showed that loss of membrane integrity as indicated by PI staining was the final signal after decrease of all other tested physiological functions. In a second study, Berney at al. [5] used PI staining for analyzing the microflora of a set of drinking water samples. The viability of the drinking water bacteria was higher for bottled water (about 90%) and drinking fountain water (about 85%) than for drinking water at the tap (about 66%). The high percentage of viable cells coincided with a high ATP content. The comparison of PI staining with other methods demonstrated PI staining was a valuable criterion for live–dead distinction for drinking water bacteria.

Autofluorescence is a feature that has to be taken into account as a potentially misleading signal for the analysis of aquatic bacterial communities by PI/SYTO9 staining [39]. According to our taxonomic analyses, two phylotypes were affiliated with the phylum *Chloroflexi* whose members are known to contain bacteriochlorophyll c and a in the chlorosomes and the cytoplasmic membrane resulting in green autofluorescence [28]. The *Chloroflexi* were detected in the membrane-intact and membrane-injured sorted fractions, but with a far higher detection in the membrane-intact fractions (up to 23% for PT 24 in the RNA-based analyses). In the latter case a wrong "live" sorting due to the autofluorescence cannot be ruled out. On the other hand, a false "dead" sorting could have been caused by phylotypes affiliated with the genus *Synechococcus* due to the presence of red fluorescent phycoerythrin [37]. Phylotype 46 that was common in the RNA- and DNA-based analyses and closely related to *Synechococcus rubescens* had a high abundance in the "dead" sorting of 10% for the DNA and of 15% for the RNA-based analysis, respectively. Though autofluorescence may be misleading for the live–dead sorting of some bacteria with photosynthetic pigments, we do not consider this as a critical issue for the live/dead staining procedure as a distinction for drinking water bacteria. Autofluorescent bacteria are commonly not considered as pathogenic and therefore, autofluorescence does not seem a critical issue for our staining procedure in respect to human health.

Live and Dead Assessment of Different Phyla and Phylotypes

All DNA-based phylotypes and 90% of the RNA-based phylotypes were retrieved after sorting in the membrane-intact and/or membrane-injured fraction. The three missing RNA phylotypes might have been missed due to their low abundance in the tap water. This close to complete recovery of the phylotypes after sorting allows a comparison of the sorting results between the DNA- and RNA-based analyses. Though the sequencing success was 77% for the RNA-based analyses and only 57% for the DNA-based analyses, the comparison can be done on the level of the retrieved phylotypes that indeed had a relatively high abundance compared to the not-retrieved phylotypes.

A comparison shows that the phylotypes of the DNA-based analyses had the same size of the "dead fraction" as those reflected by the RNA-based analyses, i.e., 21%. Also, the DNA and RNA phylotypes had a comparable percentage of only "live" sorted (DNA, 38%; RNA, 32%) and of "mixed" sorted phylotypes (DNA, 42%; RNA, 46%). Phylotype 4 concomitantly retrieved from DNA and RNA analyses was recovered from membrane-intact and membrane-injured fractions in the DNA- and RNA-based analysis, i.e., for the only common phylotype comparable sorting results were obtained for the DNA and RNA-based analysis. The second common phylotype (PT 46) cannot be compared due to the potential interference with the pigments (see above). Based on our observation, we can say that the fraction of phylotypes with only membrane-injured cells is not higher for the bacteria reflected by the DNA analyses than those of the RNA analyses. This is an essential finding because it was often assumed that those reflected by the RNA are alive, and those reflected by the DNA are dead [14]. Based on this observation, we assume that the reason for the detection of a phylotype in the DNA- or RNA-based analyses might be the phylotype-specific

regulation of the DNA and the RNA pool and was obviously not related to the viability of the respective phylotypes. This is consistent with analyses of [25] showing a broad range of numbers of rRNA operons (1–13) specific for each bacterial strain. On the other hand, we observed that all fingerprints of the membrane-intact fractions showed rather similar RNA-based fingerprints reflecting actively growing members of the community. This tight clustering of the RNA-based fingerprints from live bacteria could indicate that always the same actively growing members of the drinking water community re-grew after chlorination had killed most bacteria during water processing. This is no contradiction to the detection of a substantial amount of RNA-based phylotypes in the dead fraction because several of the live RNA phylotypes were different from the dead ones (phylotype 12, 24) or were abundant in different amounts (Fig. 6b, Supplementary Table 2b). These dead RNA phylotypes could still be remnants of the highly active phylotypes before chlorination which have not re-grown. Overall, we think that the combination of FACS sorting and fingerprinting is a promising way to obtain "functional fingerprints"—with the live RNA phylotypes representing the most actively growing members of the microbial community [12, 30].

Taxonomic Composition of the Bacterial Community of Drinking Water and Human Health

The bacterial community was composed of seven phyla (see Supplementary Material Tables 1 and 2). The phyla as well as the phylotypes are primarily those typically present in aquatic ecosystems [14, 40]. However, one phylotype detected in the drinking water had the potential of being an opportunistic pathogen. The alphaproteobacterium PT 14 identified as closely related to *B. vestrii* in the RNA-based analysis was retrieved from the membrane-intact and membrane-injured sorted fraction, and was present in the drinking water at a high abundance of 13%. This species was occasionally associated with infections of immuocompromised people [26].

However, the mere detection of a bacterium at a taxonomic resolution close to the species level is not sufficient as an indication of a health risk. Presence, viability, and infectivity of pathogenic bacteria in drinking water are criteria that have to be fulfilled for assessing a threat to human health. Presence of bacteria can be assessed by the applied technology to the detection limit of the method which is about 0.1% of the total microflora. Viability was assessed by the live/dead staining. Infectivity asks first for the precise taxonomic identification of the pathogen and a separate, mostly experimental, assessment of infectivity that has to be achieved in addition to 16S rRNA gene based analyses. Concerning the precise assessment of the taxonomy, the about 400-nt-long sequences obtained from a SSCP gel can resolve, at best, the species level. Though this accuracy might be highly valuable for the study of environmental bacteria, for most pathogenic bacteria, a full (>1,400 nt) 16S rRNA sequence is needed or even a high-resolution genotyping as exemplified for *Legionella pneumophila* in drinking water [23] or gene seuqences associated with infectivity of the respective species (e.g., the *mip* gene for *L. pneumophila)*. Thus, the proposed technology can provide a valuable monitoring tool that can show that a potentially harmful species is present—but it remains with the "potential" and the true risk has to be assessed consecutively by additional adequate measurements.

Conclusion

In summary, the approach used in this study is considered a valuable tool for analyses of aquatic bacterial communities. The applied PI/SYTO9 staining procedure indicating membrane injury of the bacterial cells is considered as a reliable criterion for damaged or dead bacterial cells. The combined approach of DNA- and RNA-based fingerprint analyses with live–dead staining and sorting was demonstrated as a straight forward monitoring tool. This tool still can be modified and extended with respect to sensitivity or methodological details. For example, in terms of methodology, PI/SYTO9 stain could be replaced by propidium monoazide application thereby avoiding the step of FACS sorting [32]. On the other hand, the sorted cells can be submitted to further labeling/staining and subsequent analyses. For increased sensitivity with respect to specific groups of pathogenic relevance, the general bacterial primers (COM1, 2) could be replaced by group-specific primer reaching a lower detection limit and a better taxonomic resolution of the targeted group. Thus, the approach can be used for monitoring of bacteria relevant to human health and can be applied as a valuable tool for drinking water monitoring with respect to the overall community or specific target pathogenic bacteria.

From an ecological perspective, the study provided comprehensive insights into the community composition and the viability of drinking water bacteria and shows that a very different spectrum of species was detected by DNA- and RNA-based analysis. A major finding in ecological terms is the fact that the viability of the phylotypes was comparable for RNA and DNA extracts. The viability of the phylotypes in addition to the very different spectrum of species detected (included pathogenic ones) demonstrate the value of adding RNA-based analyses to the commonly applied DNA-based analyses for drinking water studies or, in more general terms, for aquatic studies.

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